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(54) Title: A PROCESS FOR PRODUCING POLYPEPTIDES WITH REDUCED ALLERGENICITY

(57) Abstract

The invention relates to a process for producing polypeptides with reduced allergenicity, by a) fermenting a microorganism capable of producing said polypeptide, b) recovering said polypeptide in substantially pure form, wherein said microorganism is modified in a manner whereby the expressed polypeptide molecules self-oligomerize. Further contemplated are a DNA construct comprising genes encoding such polypeptides, a recombinant expression vector or transformation vehicle comprising said DNA construct, a cell harbouring said DNA construct or vector. Further microbially produced polypeptides with reduced allergenicity produced according to the process of the invention and compositions comprising said polypeptides. Finally the invention relates to the use of Zipper domains for reducing allergenicity of polypeptides.

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**Title:** A process for producing polypeptides with reduced allergenicity

## FIELD OF THE INVENTION

The invention relates to a process for producing polypeptides with reduced allergenicity. Further to a DNA construct comprising genes encoding such polypeptides, a recombinant expression vector or transformation vehicle comprising said DNA construct, a cell harbouring said DNA construct or vector. Also contemplated is polypeptides with reduced allergenicity produced according to the process of the invention, oligomeric polypeptides with reduced allergenicity, and compositions comprising said polypeptides. Finally the invention relates to the use of Zipper domains for reducing the allergenicity of polypeptides.

## BACKGROUND OF THE INVENTION

An increasing number of polypeptides, including enzymes and non-enzymatic proteins, are being produced industrially for use in industry, household, food/feed, cosmetics, medicine etc. Being polypeptides they are capable of stimulating the immune systems of animals and humans.

Allergenicity of polypeptides

Generally, most humans are not exposed to polypeptides to a degree that will generate adverse effects, but certain risk groups exist for which these phenomena are of significant importance.

These risk groups include employees handling the manufacturing products comprising enzymes and professions such as hairdressers which are daily in direct contact with products comprising polypeptides.

For such risk groups certain polypeptides can elicit the production of different kinds of antibodies and/or give a cellular

response. At least one of these routes can give adverse effects in humans and animals, as exposur to polypeptides may result in sensitisation and subsequently allergy.

While sensitisation is defined as an immune status, allergy is characterized as a clinical disease. Allergy in general requires two or more encounters with antigens. The first exposure lead to a primary immune response which results in sensitisation of the individual. If the sensitised individual comes in contact with the same antigen again it may provoke an allergic response.

More specifically, IgE (or comparable molecules) attach to specific receptors on the surface of mast cells, which contain many large cytoplasmic granules packed with chemical mediators. Once attached to a mast cell, the IgE molecule can survive for many weeks with its antibody reaction site, available to interact with a specific allergen.

Individuals having IgE-mediated allergy have many IgE antibodies fixed to mast cells. Upon exposure the specific allergen molecules readily combines with the cell-fixed corresponding IgE antibodies. This leads to cellular release of the cytoplasmic granules of chemical mediators, which gives symptoms like rhinitis, conjunctivitis, uricaria or other allergic reactions.

Such allergic responses occur within minutes or a few hours after exposure to an allergen, and are often referred to as "immediate hypersensitivity reactions".

IgE-mediated hypersensitivity reaction may occur when the allergen is introduced via the respiratory tract by inhalation.

The occurrenc of allergic responses is believed at least partly to depend on the way of exposure. For instanc , it have been found that intranasal challenge with allergenic proteins provokes an allergic response even though skin tests and radioallergosorbent test (RAST) for specific serum IgE are

negative (Ivan Roitt, "Essential Immunology", fifth edition, p. 152 and p. 240, 1984).

Reduction of allergenicity of polypeptides

In general prior art methods for reducing the allergenicity of polypeptides consist of various ways of immobilizing, granulating, coating or dissolving the polypeptides to avoid especially polypeptides in dust form from stimulating the immune system.

There will anyhow still be a risk of having polypeptide dust or dissolved polypeptide in aerosol form. Therefore some release of polypeptides can occur leading to a possible sensitisation and subsequent allergic response.

Another way of diminishing the problem has been to select polypeptides of human origin for production, e.g. in bacteria or mammalian cell cultures. This may alleviate some problems for humans, but not for animals. Furthermore, it will in many cases not be possible to find polypeptides of human origin with the desired properties, for which reason other origin has to be considered. This can be either human polypeptides that are altered in one or more positions in the molecule, giving performance that is desired. It might also be molecules from other species, including bacteria, mold etc. All the latter groups of products will have potency for immune stimulation.

A further proposition for decreasing allergenicity has been to reduce the size of the polypeptide molecules (see e.g. JP Patent Publication No. 4112753, or Research Disclosure No. 335102). This is, however, a solution that is only available when the activity of the polypeptide is without importance, or in such rare cases, where the activity of the polypeptide in question is retained in spite of a breakdown of the polypeptide.

The use of protein engineering has been suggested to reduce the allergenicity of polypeptides through epitope mapping and sub-

sequent change of the allergenic epitopes (see WO 92/10755 (Novo Nordisk A/S).

In the medicinal field suggestions have been made of diminishing the antigenicity or immunogenicity of polypeptides through the attachment of one or more polymeric molecules to the polypeptide. This usually has the effect of interfering with the interactions of the polypeptide with other macromolecular structures.

Such a conjugate may also exhibit novel properties: e.g. EP 38 154 (Beecham Group Ltd.) discloses conjugates of allergens with polysarcosine which have immunosuppressive properties.

US patent no. 4,179,337 (Enzon) concerns non-immunogenic polypeptides, such as enzymes and peptide hormones coupled to polyethylene glycol (PEG) or polypropylene glycol. Between 10 and 100 moles of polymer are used per mole polypeptide and at least 15% of the physiological activity is maintained. The protected polypeptide is injected in an aqueous solution either into the mammalian circulatory system or intramuscularly. The non-immunogenicity is assessed from intradermal injection tests.

It has been found that the attachment of one or more polymeric molecules to a polypeptide molecule in general has the effect of reducing the activity of e.g. the enzyme or interfering with the interaction between the enzyme and its substrate.

EP 183 503 (Beecham Group PLC) discloses a development of the above concept by providing conjugates comprising pharmaceutically useful polypeptides linked to at least one water-soluble polymer by means of a reversible linking group.

EP 471 125 (Kanebo, LTD.) d scribes a modified protease linked to a polysaccharide via a triazine ring leading to a suppressing effect on antigenicity and dermal hypersensitivity. The employed polysaccharide has an average molecular weight not less than 10,000. The modification rate for surface amino acid

groups in the modified protease is not less that 30%.

In general it is believed that allergens, entering the respiratory tract, must have a molecular weight lower than about 100 kDa in order to penetrate the plasma membrane and cause allergic reactions.

WO 94/10191 (Novo Nordisk A/S) discloses a process for production of low allergenic protein, wherein the monomeric parent protein molecules are linked together to form an oligomer. This is done *e.g.* by using a linker or spacer molecule or by linking the monomeric molecules together by peptide bonds between the C-terminal of the first monomer and the N-terminal of the second monomer.

Folkeson et al., Acta Physiol. Scand, 139, p. 437-354, 1990, showed that there is an inverse relationship between the molecular weight of an instilled protein marker and the transferred amount (bioavaibility) via the respiratory tract to the blood stream.

EP 215 662 (Masda, Hiroshi) concerns a modified or unmodified protease derived from microorganisms for use in medicaments such as anti-tumour agent. The modification of the protease may be carried out by forming dimers or oligomers by cross-linking the protease molecules.

Enlargement of polypeptides by the use of Zipper domains

As can be seen from the above different techniques of enlarging polypeptides have been known for some time.

"Zipper technique"

Another technique which may be referred to as "Zipper technique" is known to cause oligomerization of polypeptides.

Zipper technique makes it possible to link polypeptide molecules to each other by means of self-oligomerizing polypeptide

domains (in the following referred to as Zipper domains). Examples of such Zipper domains include the well-known α-helical bundles, crossed bundles, multiple bundles, parallel coiled coils, poly(L-glutamine) strands.

The simplest cases of Zipper domains include the helical bundles which consist of amphiphilic helices, e.g. Leucine Zippers and four α-helical bundles. These domains share a characteristic seven-amino acid repeat of the type $(a,b,c,d,e,f,g)_n$. Positions "a" and "d" of the heptad repeat are generally hydrophobic, a property which signals the potential for the interlocking of α-helices, as suggested by Crick (Acta crystallogr., 6, p. 689-697, 1953).

Despite the common pattern different sequences form two-, three, four-stranded and even higher order stranded helical bundles (Cohen et. al., TIBS, 11, 245-248, 1986; Cohen, Proteins, vol. 7, p. 1-15, 1990; Cohen, Science 263, p. 488-489, 1994; O'Shea et al., Cell, 68, p. 699-708, 1992; O'Shea et al., Science, 254, p. 539-545, 1991; O'Shea et al. Science, 243, p. 538-542, 1989; Eisenberg et al., Proteins, 1, p.16-22, 1986; Ho et al., J. Am. Chem. Soc, 109, p. 6751-6758, 1987).

An example of a Leucine Zipper is the 33 amino acid sequence located at the C-terminus of GCN4, a yeast transcription factor, which belongs to a class of DNA binding polypeptides (O'Shea et al. Science, 243, p. 538-542, 1989).

Through genetic engineering the specific GCN4 Leucine Zipper has been fused to different polypeptides and shown to mediate dimerization of monomeric polypeptides.

Hu et al., Science, Vol. 250, p. 1400-1403, 1990, describes a genetic system where a GCN4 Leucine Zipper is fused to the N-terminal domain of bacteriophage λ repressor and used as a reporter for dimerization.

Blondel and Bedouelle (Protein Engineering, 4, p. 457-461,

1991) dimerized a maltose binding protein (MalE) in *E. coli*.

In general Leucine Zippers form homo-dimers, but within the group of Leucine Zippers there are specific motifs which favour the formation of hetero-dimers. Two examples of such are the Fos and Jun Leucine Zipper (O'Shea et al. Science, 245, p. 646, 1989; Turner and Tjian, Science, 243 p. 1689, 1989) and the artificially made hetero dimeric coiled coil described by O'Shea (Current Biology, vol. 3, no 10, p. 658-667, 1993).

Also the above mentioned self-oligomerizing four α-helical bundles have been shown to dimerize with a murine ScFv antibody fragment expressed in *E. coli*. The antibody fragment is fused to polypeptide motifs (see figure 7) of two identical helices from antiparallel four helical bundle designed by Eisenberg et al., supra, 1986 and Ho et al., supra, 1987, in which the two helices are separated by a turn. The four helix bundle was formed from two molecules each contributing two helices (Pack et al. Bio/Technology, vol. 11, p. 1271-1277, 1993).

Concerning Zipper domains resulting in higher order oligomerization, Lovejoy et al., Science, 259, p. 1288, 1993, reported the synthesis of a triple stranded α-helix bundle in which the helices run up-up-down. This construct was made by introducing specific mutations in the otherwise dimerized GCN4 Leucine Zipper.

Incorporation of glutamine repeats (poly(L-glutamine) also makes proteins oligomerize to form polar Zippers (Stott et al., (1995), Proceedings of the National Academy of Sciences of the United States of America 92 (14), p. 6509-6513.

Another polypeptide motif which can mediate trimerization is the naturally occurring motif of the shock transcription factor of *Saccharomyc s cer visiae* and *Kluveromyces lactis* described by Peteranderl et al, Biochemistry, 31, p. 12272-12276, 1992.

Examples of tetrameric formation involves altering amino acid

residues in the GCN4 Leucine Zipper (Harbury et al., Science, 262, p. 1401-1407, 1993. This was only shown for small peptides.

An oligomerization motif from myosin fused to recombinant protein can mediate the formation of higher order polypeptide oligomers (Wolber et al., BIO/TECHNOLOGY, 10, p. 900-904, 1992). The expressed fusion polypeptide forms oligomers at low salt concentration and dissociates at high salt concentrations.

Discussion of prior art

All the previously described methods for reducing the allergenicity of polypeptides involve at least one additional production step in comparison to the production of the corresponding parent polypeptides. This makes the processes cumbersome and raises the cost of producing polypeptides with reduced allergenicity.

Prior art only describes Zipper domains as means for oligomerizing polypeptides.

It would be desirable to be able to reduce the allergenicity of polypeptides by increasing the size of the polypeptides as an integrated part of the polypeptide production process.

## SUMMARY OF THE INVENTION

It is the object of the invention to provide an integrated industrial applicable process for producing polypeptides with reduced allergenicity.

The present inventors have perceived the potential of using Zipper domains for industrial uses, and have now surprisingly accomplished to provide a process for producing a polypeptide with reduced allergenicity, by

a) fermenting a microorganism capable of producing said

polypeptide, and

b) r covering said polypeptide in substantially pure form, wherein said microorganism is modified in a manner whereby the expressed polypeptide molecules self-oligomerize.

In an embodiment of the invention said microorganism has been modified by the introduction of one or more DNA constructs comprising a DNA sequence coding for at least one polypeptide and at least one Zipper domain.

Another object of the invention is to provide a DNA construct for the production of polypeptides with reduced allergenicity, comprising a DNA sequence encoding at least one polypeptide molecule with at least one Zipper domain.

The invention also relates to a recombinant vector or transformation vehicle, comprising said DNA construct of the invention, and furthermore to a cell comprising said DNA construct or said recombinant vector or transformation vehicle.

Further, the invention is directed towards microbially polypeptides with reduced allergenicity produced according to the process of the invention. Also contemplated are compositions comprising at least one polypeptide component of the invention.

Finally the invention relates to the use of Zipper domains for reducing the allergenicity of polypeptides.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 shows the DNA sequence and derived amino acid sequence of a linker, the GCN4 Leucine Zipper and a flexible C-terminal extension peptide containing a cystein amino acid residue.

Figure 2 shows the construction steps of the pAZ-1 plasmid.

Figure 3 shows a coomassie blue stained SDS-PAGE run under non-reducing conditions, wherein lane 4 is a molecular weight marker SeeBlue™ (Cat.:#LC5625, Novex, Inc., Ca, USA), and lanes 2 and 3 are polypeptides expressed by *E. coli* JM105 comprising the DNA construct pAZ-1.

Figure 4 shows coomassie blue stained SDS-PAGE run under reducing conditions, wherein lane 1 is a molecular weight marker SeeBlue™ (Cat.:#LC5625, Novex, Inc., Ca, USA), and lanes 2 and 3 are polypeptides expressed by *E. coli* JM105 comprising the DNA construct pAZ-1.

Figure 5 shows a Western blot. Lane 1 is the molecular weight marker. Lanes 2 and 3 are samples from the induced JM105/pAZ-1 run under non-reduced conditions. Lanes 4 and 5 are the same samples run under reduced conditions.

Figure 6 shows the number of Dunkin Hartley guinea pig, having been exposed to 1.0 µg monomer and 1.0 µg dimer Termamyl® intratracheally, found to be $IgG_1$ positive vs. days starting from the day of exposure.

**DETAILED DESCRIPTION OF THE INVENTION**

The present inventors have now surprisingly succeeded in providing an integrated industrially applicable process for producing polypeptides with reduced allergenicity, wherein the biological activity of the polypeptides is at least substantially maintained.

A "substantially" maintained activity is in the context of the present invention defined as an activity which is at least between 20% and 30%, preferably between 30% and 40%, more preferably between 40% and 60%, better from 60% up to 80%, even better from 80% up to about 100%, in comparison to the activity of the not modified parent polypeptide.

Said polypeptides may be used for a vast number of industrial applications which will be exemplifi d below.

It is to be understood that, in connection with industrial applications of polypeptides, it is mainly inhalation of the allergens that may inflict a risk of an allergic response. Therefore, one of the crucial advantages of the present invention is, that the inventors have solved the problem of respiratory challenge with allergens, whereas prior art solutions mainly concern dermal challenge with alleged immunogens. Respiratory challenge is a much more sensitive question.

The term "reduced allergenicity" indicates that the amount of produced IgE (in humans, and molecules with comparable effects in specific animals, for instance $IgG_1$ in guinea pigs), which can lead to an allergic state are significantly decreased when inhalating a polypeptide of the invention in comparison to the corresponding parent polypeptide.

The terms "immunogen", "antigen" and "allergen" are defined below, as these term often are use in an unclean manner, even by scientists.

An "immunogen" may be defined as a substance which when introduced into humans and animals is capable of stimulating an immunologic response.

The term "antigen" refers to substances which by themselves are capable of generating antibodies when recognized as a non-self molecule by the immune system.

Further, an "allergen" may be defined as an antigen which may give rise to allergic sensitization or an allergic response by IgE antibodies (in humans, and molecules with comparable effects in animals).

It is to be understood that the term "immunogen" is th wider term and includes "antigen" and "allergen".

As mentioned above it is, at 1 ast in the context of polypeptides of the present inv ntion, important to distinguish between dermal allergens mediating allergic responses caused by skin contact, and respiratory allergens causing allergic responses by contact with cell-bound IgE in the bronchial tree, due to the well-known fact that skin tests may be negative even though inhalation tests provoke an allergic response.

Therefore assessment of allergenicity may be made by inhalation tests, comparing the effect of intratracheal administrated parent polypeptides with the corresponding polypeptides of the invention with reduced allergenicity.

Two main hazard assessment approaches exists, animal models and *in vitro* models, respectively. Animal models recommended by ECETOC (see Monografi ECETOC no. 19, p. 17-27) includes both mice and guinea pig models.

The mice models focus upon events occurring during the induction phase of sensitisation following primary encounter of the substance in question. However, mice are not considered suitable for investigating polypeptides.

In contrast hereto the guinea pig models seek to identify respiratory allergens as a function of elicitation reactions induced in previously sensitised animals. ECETOC assesses results of studies using guinea pig as a suitable basis for hazard assessment in man.

Specifically, in the context of assessment of allergenicity of polypeptides according to the invention, models involving introduction of polypeptides intratracheal in guiniea pigs are suitable.

One suitable strain of guinea pigs, the Dunkin Hartley strain, does not (as humans) produce IgE antibodies in connection with the allergic response. However, they produce another type of antibody the $IgG_1A$ and $IgG_1B$ which characterize their allergenic

respons to inhaled polypeptides (see *e.g.* Prentø, ATLA, 19, p. 8-14, 1991).

Therefore when using the Dunkin Hartley animal model, the relative amounts of $IgG_1A$ and $IgG_1B$ are a measure for the allergenicity level.

Other animal models such as rats, rabbits etc. could also be used for comparable studies.

The production of a polypeptide with reduced allergenicity according to the invention specifically comprises

a) fermenting a microorganism capable of producing said polypeptide, and

b) recovering said polypeptide in substantially pure form, wherein said microorganism is modified in a manner whereby the expressed polypeptide molecules self-oligomerize.

The allergenicity of the polypeptides is believed to be reduced by the enlargement of the polypeptides.

The term "self-oligomerization" does in the context of the present invention mean joining together a desired number of polypeptide molecules, *e.g.* by the use of Zipper domains, and includes dimerization, trimerization, tetramerization, multimerization, polymerization etc.

In a preferred embodiment of the invention the microorganism is modified by introducing one or more DNA construct(s) into said microorganism. Said DNA construct comprises a DNA sequence encoding at least one polypeptide of interest operably linked to at least one Zipper domain. Optionally the DNA sequence may further comprise a short linker sequence between the sequence encoding the polypeptide and the Zippper domain and/or a DNA sequence encoding a purification tag.

The recovery of the oligomerized polypeptide may be carried out in any suitable way. In the case of using a poly-His tail

purification tag the polypeptides may be r covered by IMAC (Immobbilized Metal Affinity Chromatography) following the procedure described in e.g. Yip et al., (1994;, Molecular Biotechnology, vol. 1, p. 151-164; Fatiadi et al., (1987), CRC Critical Rev. Anal. Chem. 18, p. 1-44.

A linker sequence is a DNA sequence encoding an amino acid sequence connecting the polypeptide in question and the Zipper domain.

The enlargement of the polypeptides takes place during fermentation. The amino acid sequence of the Zipper domain is expressed grafted to the N- or C-terminal of the polypeptide of interest. When this fusion-polypeptide is expressed Zipper domains associate e.g. two and two and are held together by hydrophobic and electrostatic interactions.

Dependent on the Zipper domain used the fusion polypeptide may also form trimers, tetramers etc.

The process of the invention is advantageous due to the fact that no additional step need to be executed after the fermentation and before the recovery to obtain the polypeptide product with reduced allergenicity.

Further, it is also an advantage that the process according to the invention may be used for any polypeptides of interest, which may be any polypeptides that in parent form may cause an allergic reaction.

This group comprises polypeptides having a molecular weight below about 100 kDa. In general the said molecular weight lies in the range of between about 5 kDa and 150 kDa, preferably from between about 20 kDa and 100 kDa, especially from between about 20 kDa and 80 kDa.

Th polypeptides may be of microbial or mammalian origin and may be naturally occurring polypeptides or variants thereof.

In an embodiment of the invention th polypeptide of interest is an enzyme exhibiting at least one catalytic activity.

Such enzymes may be selected from the group comprising proteases (metallo, acid, neutral or alkaline), lipases, cutinases, cellulases, amylases, lyases, xylanases, pectinases, pullulanase, polygalacturonases, oxidases, laccases, oxidoreductases, transglutaminases, α-galactocidases, phytases and peroxidases

A specific example of such enzyme is Termamyl® (Novo Nordisk A/S), an α-amylase, having a molecular weight of about 55 kDa. The process of the invention enables the production of hybrid products exhibiting more than one biological activity, *e.g.* hetero-dimeric enzymes which exhibit two different catalytic activities, such as lipolytic and proteolytic activities.

Also contemplated are trimeric, tetrameric, multimeric polypeptides and/or enzymes exhibiting one or more catalytic activities.

The polypeptide with reduced allergenicity may be produced by any suitable bacteria or fungal organisms as described below.

DNA construct

Another object of the invention is to provide a DNA construct for the production of polypeptides with reduced allergenicity, comprising a DNA sequence encoding at least one polypeptide, and at least one Zipper domain.

As used herein the term "DNA construct" is intended to indicate any nucleic acid molecule of cDNA, genomic DNA, synthetic DNA, RNA or PNA origin. The term "construct" is intended to indicate a nucleic acid segment which may be single- or double-stranded, and which may b based on a DNA sequence encoding a polypeptide of interest fused to at least one Zipper domain. The construct may optionally contain other DNA segments, such as a short linker sequence and/or a sequenc encoding a peptide segment specifically used for purification purposes.

The DNA construct of the invention may suitably be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or parts of the polypeptide of interest by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY, 1989).

The DNA sequence may encode a polypeptide exhibiting catalytic activities. In particular, the DNA sequence may encoding at least one enzyme selected from the group comprising proteases (metallo, acid, neutral or alkaline), lipases, cutinases, cellulases, amylases, lyases, xylanases, pectinases, polygalacturonases, oxidases, laccases, oxidoreductases, transglutaminases, α-galactosidases, phytases or peroxidases.

The DNA construct of the invention may also be prepared synthetically by established standard methods, *e.g.* the phosphoamidite method described by Beaucage and Caruthers, Tetrahedron Letters, 22, p. 1859 - 1869, 1981, or the method described by Matthes et al., EMBO Journal, 3, p. 801 - 805, 1984. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

Furthermore, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA construct, in accordance with standard techniques.

The DNA construct may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or Saiki et al., Science, 239, p. 487 - 491, 1988.

In a specific embodiment the DNA construct of the invention comprises the DNA sequence shown in SEQ ID NO 1 as well as

nucleic acid sequences encoding the amino acid sequence shown in SEQ ID NO 2, but may differ from the DNA sequence shown in SEQ ID NO 1 by virtue of the degeneracy of the genetic code.

Recombinant vector

In a further aspect the present invention relates to a recombinant vector or transformation vehicle comprising a DNA construct of the invention. The recombinant vector into which the DNA construct of the invention is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the polypeptide of interest to be self-oligomerized is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide of interest.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al.,

J. Biol. Chem., 255, p. 12073 - 12080, 1980; Alber and Kawasaki, J. Mol. Appl. G n., 1, p. 419 - 434, 1982) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature, 304, p. 652 - 654, 1983) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J., 4, p. 2093 - 2099, 1985) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gen, or the *Bacillus pumilus* xylosidase gene, or by the phage Lambda $P_R$ or $P_L$ promoters or the *E. coli* lac, trp or tac promoters.

The DNA sequence may also, if necessary, be operably connected to a suitable terminator.

The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the host cell in question..

The vector may also comprise a selectabl marker, e.g. a gene th product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyc s pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, p. 125-130), or one which confers

resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, phl omycin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, trpC and sC.

To direct the polypeptide into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the polypeptide in the correct reading frame. Secretory signal sequences are commonly positioned 5′ to the DNA sequence encoding the polypeptide. The secretory signal sequence may be that normally associated with the polypeptide or may be from a gene encoding another secreted polypeptide.

For secretion from yeast cells, the secretory signal sequence may encode any signal peptide which ensures efficient direction of the expressed polypeptide into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the α-factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et al., Nature, 289, p. 643-646, 1981), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell, 48, p. 887-897, 1987), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast, 6, p. 127-137, 1990).

For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and upstream of the DNA sequence encoding the polypeptide. The function of the leader peptide is to allow the expressed polypeptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the polypeptide across the cell wall or at least through the

cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast α-factor leader (the use of which is described in e.g. US 4,546,082, EP 16 201, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase.

The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase.

In a preferred specific embodiment of the invention said vector is the pAZ-1 expression vector.

The procedures used to ligate the DNA sequences coding for the polypeptide in question, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., supra, 1989).

Host cell

The DNA sequence encoding the fusion polypeptide in question, introduced into the host cell may be either homologous or heterologous to the host in question. If homologous to the host cell, i.e. produced by the host cell in nature, it will typically b operably connected to another promoter sequence or, if applicable, another secretory signal sequence and/or terminator sequence than in its natural environment. The term "homologous" is intended to include a cDNA sequence encoding a polyp ptide native to the host organism in question. The term

"heterologous" is intended to include a DNA sequence not x-pressed by the host cell in nature. Thus, the DNA sequence may be from another organism, or it may be a synthetic sequence.

The host cell into which the DNA construct or the recombinant vector of the invention is introduced may be any cell which is capable of producing the polypeptide of interest and includes bacteria, yeast, filamentous fungi.

Examples of bacterial host cells which, on cultivation, are capable of producing the polypeptide of interest are gramposi-tive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stea-rothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thurin-giensis*, or strains of *Streptomyces*, such as *S. lividans*, *S. murinus* or *S. griseus*, or gramnegative bacteria such as *Esche-richia coli*. The transformation of the bacteria may be effected by protoplast transformation or by using competent cells in a manner known per se (cf. Sambrook et al., supra).

When expressing the polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or may be di-rected to the periplasmic space by a bacterial secretion se-quence. In the former case, the cells are lysed and the granules are recovered and denatured after which the poly-peptide is refolded by diluting the denaturing agent. In the latter case, the polypeptide may be recovered from the peri-plasmic space by disrupting the cells, *e.g.* by sonication or osmotic shock, to release the contents of the periplasmic space and recovering the polypeptide.

Examples of suitable yeasts cells include cells of *Saccharomy-c s* spp. or *Schizosaccharomyc s* spp., in particular strains of *Saccharomyc s cerevisiae* or *Saccharomyc s kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptide therefrom are described, *e.g.* in US

4,599,311, US 4,931,373, US 4,870,008, 5,037,743, and US 4,845,075, all of which are hereby incorporated by ref rence. Transformed cells are selected by a phenotype determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4,931,373. The DNA sequence encoding the polypeptide of the invention may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, p. 3459-3465; US 4,882,279).

Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of polypeptides is described in, e.g., EP 272 277, EP 238 023 and EP 184 438. The transformation of *F. oxysporum* may, for instance, be carried out as described by Malardier et al., Gene, 78, p. 147-156, 1989.

When a filamentous fungus is used as the host cell it may be transformed with the DNA construct of the invention conveniently by integrating the DNA construct in the host chromosome to obtain a recombinant host cell. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination.

The transform d host cell described above is then cultured in a suitable nutrient medium under conditions permitting the expression of the polypeptide of inter st, after which the resulting polypeptide is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection). The polypeptide produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, *e.g.* ammonium sulphate, purification by a variety of chromatographic procedures, *e.g.* ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of polypeptide in question.

Polypeptides

The invention is also directed towards polypeptides with reduced allergenicity produced according to a process of the invention.

The polypeptide of the invention consist of a Zipper domain fused to the polypeptide of interest. The N- or C-terminal of the amino acid sequence of the polypeptide is grafted to the Zipper domain.

The Zipper domain may be any domain capable of oligomerizing the polypeptides in the production phase. In a specific embodiment the Zipper domain is a Leucine Zipper, such as the GCN4 Leucine Zipper.

Preferably the monomeric polypeptide has a molecular weight of between 5 kDa and 150 kDa, preferable between 20 kDa and 100 kDa, especially between 20 kDa and 80 kDa.

When using Leucine Zipp rs for oligomerizing polypeptides consisting of two polypeptide molecul s, such as two Termamyl mol cules, the Leucine Zippers normally have enough affinity to

k ep the homo-dimer stable. However, to further stabilize the dimer, a cysteine may be included in the Leucine Zipper. This construction can lead to the formation of a disulfide bridge between the two monomers of hybrid polypeptide.

The polypeptide of the invention comprises from 2 to 10 polypeptide molecules or more. In a preferred embodiment the self-oligomerized polypeptide is a dimer, a trimer, a tetramer, or an oligomer.

It is possible to persist or resume the biological activity of the polypeptide, *e.g.* the enzymatic activity of a dimerized enzyme.

Further the polypeptide of the invention may exhibit more than one biological activity, *e.g.* two or more different enzymatic activities, such as lipolytic and proteolytic activities.

Oligomeric polypeptides

The invention also related to oligomeric polypeptides with reduced allergenicity comprising at least one polypeptide bonded or linked to at least one Zipper domain which is coupled to at least one polypeptide bonded or linked to at least one Zipper domain.

Said oligomeric polypeptides may be homo-oligomeric, hetero-oligomeric or higher order oligomeric polypeptide produced by any suitable process or prepared by any suitable method.

Said Zipper domain may be any of the previously mentioned Zipper domains.

In an embodiment said oligomeric polypeptide exhibits at least on of the pr viously mentioned enzymatic activities.

Said Zipper domain may be linked to either the C- or N-terminal of the polypeptide(s) in question.

In the following the term "polypeptide" includes both polypeptides produced according to the process of the invention and said oligomeric polypeptides of the invention.

A polypeptide according to the invention may demonstrate a high degree of controlled stability.

In certain cases the polypeptides may advantageously be irreversible fused together, which entails that the product has only negligible tendency to disintegrate, which would lead to the return of conditions that may cause an allergenic state.

However in certain other cases, it is advantageous that the polypeptides stay oligomerized in the production and/or bulk handling phase, but dissociates later on, when the polypeptides does not inflict a risk of exposure to humans or animals.

The cleavage of the linkage between the polypeptides may be activated *e.g.* by physical conditions, such as pH, ionic strength, temperature, reduction or oxidation potential etc.

Further the presence of specific compounds may result in dissociating *e.g.* into lower order oligomers or monomers.

Especially in the case where the activity of the polypeptides are reduced in the oligomerized form, dissociation may be advantageous.

Composition

The invention also relates to a composition comprising at least one polypeptide and/or at least one oligomeric polypeptide of the invention.

The composition may further comprise other ingredients normally us d in .*g*. detergents, including soap bars, household articles, agrochemicals, personal care products, cosmetics, toiletry, pharmaceuticals, composition used for tr ating textiles, food and/or feed etc.

Detergent compositions

According to the invention, a polypeptide of the invention may be an enzyme used in detergent compositions. It may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzenesulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonat s (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol thoxylate (AEO or AE),

carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycosid , alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more enzymes, such as e.g. amylases, lipases, cutinases, proteases, cellulases, peroxidases, and oxidases.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a $H_2O_2$ source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

The detergent composition of the invention comprising the polypeptide of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as

described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

| | |
|---|---|
| - linear alkylbenzenesulfonate (calculated as acid) | 7 - 12% |
| - alcohol ethoxysulfate (e.g. $C_{12-18}$ alcohol, 1-2 EO) or alkyl sulfate (e.g. $C_{16-18}$) | 1 - 4% |
| - alcohol ethoxylate (e.g. $C_{14-15}$ alcohol, 7 EO) | 5 - 9% |
| - sodium carbonate (as $Na_2CO_3$) | 14 - 20% |
| - soluble silicate (as $Na_2O,2SiO_2$) | 2 - 6% |
| - zeolite (as $NaAlSiO_4$) | 15 - 22% |
| - sodium sulfate (as $Na_2SO_4$) | 0 - 6% |
| - sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$) | 0 - 15% |
| - sodium perborate (as $NaBO_3.H_2O$) | 11 - 18% |
| - TAED | 2 - 6% |
| - carboxymethylcellulose | 0 - 2% |
| - polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) | 0 - 3% |
| - enzymes | 0 - 5% |
| - minor ingredients (e.g. suds | |

suppressors, perfume, optical brighten r, photobleach) 0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid) 6 - 11%
- alcohol ethoxysulfate (e.g. $C_{12-18}$ alcohol, 1-2 EO) or alkyl sulfate (e.g. $C_{16-18}$) 1 - 3%
- alcohol ethoxylate (e.g. $C_{14-15}$ alcohol, 7 EO) 5 - 9%
- sodium carbonate (as $Na_2CO_3$) 15 - 21%
- soluble silicate (as $Na_2O,2SiO_2$) 1 - 4%
- zeolite (as $NaAlSiO_4$) 24 - 34%
- sodium sulfate (as $Na_2SO_4$) 4 - 10%
- sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$) 0 - 15%
- carboxymethylcellulose 0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) 1 - 6%
- enzymes 0 - 5%
- minor ingredients (e.g. suds suppressors, perfume) 0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid) 5 - 9%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO) 7 - 14%
- soap as fatty acid (e.g. $C_{16-22}$ fatty acid) 1 - 3%
- sodium carbonate (as $Na_2CO_3$) 10 - 17%
- soluble silicate (as $Na_2O,ciao_2$) 3 - 9%
- zeolite (as $NaAlSiO_4$) 23 - 33%

- sodium sulfate (as $Na_2SO_4$) 0 - 4%
- sodium perborate (as $NaBO_3.H_2O$) 8 - 16%
- TAED 2 - 8%
- phosphonate (e.g. EDTMPA) 0 - 1%
- carboxymethylcellulose 0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) 0 - 3%
- enzymes 0 - 5%
- minor ingredients (e.g. suds suppressors, perfume, optical brightener) 0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- linear alkylbenzenesulfonate (calculated as acid) 8 - 12%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO) 10 - 25%
- sodium carbonate (as $Na_2CO_3$) 14 - 22%
- soluble silicate (as $Na_2O,2SiO_2$) 1 - 5%
- zeolite (as $NaAlSiO_4$) 25 - 35%
- sodium sulfate (as $Na_2SO_4$) 0 - 10%
- carboxymethylcellulose 0 - 2%
- polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG) 1 - 3%
- enzymes 0 - 5%
- minor ingredients (e.g. suds suppressors, perfume) 0 - 5%

5) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid) 15 - 21%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO or $C_{12-15}$ alcohol, 5 EO) 12 - 18%

- soap as fatty acid (e.g. oleic acid) 3 - 13%
- alkenylsuccinic acid ($C_{12-14}$) 0 - 13%
- aminoethanol 8 - 18%
- citric acid 2 - 8%
- phosphonate 0 - 3%
- polymers (e.g. PVP, PEG) 0 - 3%
- borate (as $B_4O_7$) 0 - 2%
- ethanol 0 - 3%
- propylene glycol 8 - 14%
- enzymes 0 - 5%
- minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener) 0 - 5%

6) An aqueous structured liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid) 15 - 21%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO, or $C_{12-15}$ alcohol, 5 EO) 3 - 9%
- soap as fatty acid (e.g. oleic acid) 3 - 10%
- zeolite (as $NaAlSiO_4$) 14 - 22%
- potassium citrate 9 - 18%
- borate (as $B_4O_7$) 0 - 2%
- carboxymethylcellulose 0 - 2%
- polymers (e.g PEG, PVP) 0 - 3%
- anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800 0 - 3%
- glycerol 0 - 5%
- enzymes 0 - 5%
- minor ingredients

(e.g. dispersants, suds suppressors, perfume, optical brighteners) 0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- fatty alcohol sulfate 5 - 10%
- ethoxylated fatty acid monoethanolamide 3 - 9%
- soap as fatty acid 0 - 3%
- sodium carbonate (as $Na_2CO_3$) 5 - 10%
- soluble silicate (as $Na_2O,2SiO_2$) 1 - 4%
- zeolite (as $NaAlSiO_4$) 20 - 40%
- sodium sulfate (as $Na_2SO_4$) 2 - 8%
- sodium perborate (as $NaBO_3.H_2O$) 12 - 18%
- TAED 2 - 7%
- polymers (e.g. maleic/acrylic acid copolymer, PEG) 1 - 5%
- enzymes 0 - 5%
- minor ingredients (e.g. optical brightener, suds suppressors, perfume) 0 - 5%

8) A detergent composition formulated as a granulate comprising

- linear alkylbenzenesulfonate (calculated as acid) 8 - 14%
- ethoxylated fatty acid monoethanolamide 5 - 11%
- soap as fatty acid 0 - 3%
- sodium carbonate (as $Na_2CO_3$) 4 - 10%
- soluble silicate (as $Na_2O,2SiO_2$) 1 - 4%
- zeolite (as $NaAlSiO_4$) 30 - 50%
- sodium sulfate (as $Na_2SO_4$) 3 - 11%
- sodium citrate (as $C_6H_5Na_3O_7$) 5 - 12%
- polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG) 1 - 5%
- enzymes 0 - 5%

- minor ingredients (e.g. suds suppressors, perfum ) 0 - 5%

9) A detergent composition formulated as a granulate comprising

- linear alkylbenzenesulfonate (calculated as acid) 6 - 12%
- nonionic surfactant, 1 - 4%
- soap as fatty acid 2 - 6%
- sodium carbonate (as $Na_2CO_3$) 14 - 22%
- zeolite (as $NaAlSiO_4$) 18 - 32%
- sodium sulfate (as $Na_2SO_4$) 5 - 20%
- sodium citrate (as $C_6H_5Na_3O_7$) 3 - 8%
- sodium perborate (as $NaBO_3.H_2O$) 4 - 9%
- bleach activator (e.g. NOBS or TAED) 1 - 5%
- carboxymethylcellulose 0 - 2%
- polymers (e.g. polycarboxylate or PEG) 1 - 5%
- enzymes 0 - 5%
- minor ingredients (e.g. optical brightener, perfume) 0 - 5%

10) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid) 15 - 23%
- alcohol ethoxysulfate (e.g. $C_{12-15}$ alcohol, 2-3 EO) 8 - 15%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO, or $C_{12-15}$ alcohol, 5 EO) 3 - 9%
- soap as fatty acid (e.g. lauric acid) 0 - 3%
- aminoethanol 1 - 5%
- sodium citrate 5 - 10%
- hydrotrope (e.g. sodium toluenesulfonate) 2 - 6%
- borate (as $B_4O_7$) 0 - 2%

- carboxymethylcellulose 0 - 1%
- ethanol 1 - 3%
- propylene glycol 2 - 5%
- enzymes 0 - 5%
- minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners) 0 - 5%

11) An aqueous liquid detergent composition comprising

- linear alkylbenzenesulfonate (calculated as acid) 20 - 32%
- alcohol ethoxylate (e.g. $C_{12-15}$ alcohol, 7 EO, or $C_{12-15}$ alcohol, 5 EO) 6 - 12%
- aminoethanol 2 - 6%
- citric acid 8 - 14%
- borate (as $B_4O_7$) 1 - 3%
- polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer) 0 - 3%
- glycerol 3 - 8%
- enzymes 0 - 5%
- minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners) 0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap) 25 - 40%
- nonionic surfactant (e.g. alcohol ethoxylate) 1 - 10%
- sodium carbonate (as $Na_2CO_3$) 8 - 25%
- soluble silicates (as $Na_2O$, $2SiO_2$) 5 - 15%
- sodium sulfate (as $Na_2SO_4$) 0 - 5%

- z olite (as $NaAlSiO_4$) 15 - 28%
- sodium perborate (as $NaBO_3.4H_2O$) 0 - 20%
- bleach activator (TAED or NOBS) 0 - 5%
- enzymes 0 - 5%
- minor ingredients (e.g. perfume, optical brighteners) 0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by ($C_{12}$-$C_{18}$) alkyl sulfate .

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- ($C_{12}$-$C_{18}$) alkyl sulfate 9 - 15%
- alcohol ethoxylate 3 - 6%
- polyhydroxy alkyl fatty acid amide 1 - 5%
- zeolite (as $NaAlSiO_4$) 10 - 20%
- layered disilicate (e.g. SK56 from Hoechst) 10 - 20%
- sodium carbonate (as $Na_2CO_3$) 3 - 12%
- soluble silicate (as $Na_2O,2SiO_2$) 0 - 6%
- sodium citrate 4 - 8%
- sodium percarbonate 13 - 22%
- TAED 3 - 8%
- polymers (e.g. polycarboxylates and PVP) 0 - 5%
- enzymes 0 - 5%
- minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors) 0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

- ($C_{12}$-$C_{18}$) alkyl sulfate 4 - 8%

- alcohol ethoxylate 11 - 15%
- soap 1 - 4%
- zeolite MAP or zeolite A 35 - 45%
- sodium carbonate (as $Na_2CO_3$) 2 - 8%
- soluble silicate (as $Na_2O,2SiO_2$) 0 - 4%
- sodium percarbonate 13 - 22%
- TAED 1 - 8%
- carboxymethyl cellulose 0 - 3%
- polymers (e.g. polycarboxylates and PVP) 0 - 3%
- enzymes 0 - 5%
- minor ingredients (e.g. optical brightener, phosphonate, perfume) 0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, *e.g.*, be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature, 369, p. 637-639, 1994.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, *e.g.*, linear alkoxylated primary alcohol, a builder syst m (*e.g.* phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The enzyme of interest of the invention may be incorporated in

concentrations conventionally employed in det rgents. It is at present contemplated that, in the detergent composition of the invention, the enzyme in question with reduced allergenicity may be added in an amount corresponding to 0.001-100 mg of enzyme per liter of wash liquor.

Dishwashing composition

The polypeptides of the invention with reduced allergenicity may also advantageously be used in dishwashing detergents.

Dishwashing detergent compositions comprise a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will contain 0-90% of non-ionic surfactant such as low- to non-foaming ethoxylated propoxylated straight-chain alcohols.

The detergent composition may contain detergent builder salts of inorganic and/or organic types. The detergent builders may be subdivided into phosphorus-containing and non-phosphorus-containing types. The detergent composition usually contains 1-90% of detergent builders.

Examples of phosphorus-containing inorganic alkaline detergent builders, when present, include the water-soluble salts especially alkali metal pyrophosphates, orthophosphates, and polyphosphates. An example of phosphorus-containing organic alkaline detergent builder, when present, includes the water-soluble salts of phosphonates. Examples of non-phosphorus-containing inorganic builders, when present, include water-soluble alkali metal carbonates, borates and silicates as well as the various types of water-insoluble crystalline or amorphous alumino silicates of which zeolites are the best-known representatives.

Examples of suitable organic builders include th alkali metal, ammonium and substitut d ammonium, citrat s, succinates, malonat s, fatty acid sulphonates, carboxymetoxy succinates, ammonium polyacetates, carboxylates, polycarboxylates, amino-

polycarboxylates, polyacetyl carboxylates and polyhydroxysulphonates.

Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

The oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED and glycerol triacetate.

The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), *e.g.* a polyol such as *e.g.* propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, *e.g.* an aromatic borate ester.

The dishwashing detergent composition of the invention may also contain oth r conventional detergent ingredients, *e.g.* deflocculant material, filler material, foam depr ssors, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil r deposition agents, dehydrating agents, dyes, bacteri-

cid s, fluoresc rs, thicken rs and perfumes.

Finally, the polypetide of the invention may be used in conventional dishwashing detergents, *e.g.* in any of the detergents described in any of the following patent publications:

EP 518719, EP 518720, EP 518721, EP 516553, EP 516554, EP 516555, GB 2200132, DE 3741617, DE 3727911, DE 4212166, DE 4137470, DE 3833047, WO 93/17089, DE 4205071, WO 52/09680, WO 93/18129, WO 93/04153, WO 92/06157, WO 92/08777, EP 429124, WO 93/21299, US 5141664, EP 561452, EP 561446, GB 2234980, WO 93/03129, EP 481547, EP 530870, EP 533239, EP 554943, EP 346137, US 5112518, EP 318204, EP 318279, EP 271155, EP 271156, EP 346136, GB 2228945, CA 2006687, WO 93/25651, EP 530635, EP 414197, US 5240632.

Textile applications

Further polypeptides, including enzymes, of the invention with reduced allergenicity may be used in applications for purposes in the textile industry involving handling of enzyme granulates or powders.

Examples of textile applications are listed below:

i. Cellulolytic enzymes are widely used in the finishing of denim garments in order to provide a localized variation in the colour density of the fabric (Enzyme facilitated "stone wash").

ii. Bio-Polishing

Also cellulolytic enzymes find use in the bio-polishing process. Bio-Polishing is a specific treatment of the yarn surface which improves fabric quality with respect to handle and appearance without loss of fabric wettability. Bio-polishing may be obtained by applying the method described e.g. in WO 93/20278.

iii. Desizing

During the weaving of textiles, the threads are expos d to

considerable mechanical strain. In order to prevent breaking, they are usually reinforced by coating (sizing) with a gelatinous substance (size). The most common sizing agent is starch in native or modified form. A uniform and durable finishing can thus be obtained only after removal of the size from the fabric, the socalled desizing. Desizing of fabrics sized with a size containing starch or modified starch is preferably facilitated by use of amylolytic enzymes.

iiii. Bleach clean-up

In bleach clean-up catalases may serve to remove excess hydrogen peroxide.

iiiii. Silk degumming

Removal of gum on silk fibers with protease. (Application sheet "Novo Enzymes for Silk Degumming" is available on request)

Personal care applications

Also in the personal care technical field polypeptides according to the invention are of interest. In the following are listed examples of uses.

1) Proteases:

Proteases are well-known active ingredients for cleaning of contact lenses. They hydrolyse the proteinaceous soil on the lens and thereby makes it soluble. Removal of the protein soil is essential for the wearing comfort.

Proteases are also effective ingredients in skin cleaning products, where they remove the upper layer of dead keratinaseous skin cells and thereby makes the skin look brighter and more fresh.

Proteas s are also used in oral care products, especially for cleaning of dentures, but also in dentifrices.

2) Lipases:

Lipases can be applied for cosmetic use as active ingredients

in skin cleaning products and anti-acne products for removal of exc ssive skin lipids, and in creams and lotions as active ingredients for skin care.

Lipases can also be used in hair cleaning products (e.g. shampoos) for effective removal of sebum and other fatty material from the surface of hair.

Lipases are also effective ingredients in products for cleaning of contact lenses, where they remove lipid deposits from the lens surface.

3) Oxidoreductases:

There are many well-known Personal Care applications of oxidoreductases. The most common is an oxidase (usually glucoseoxidase) with substrate (*e.g.* glucose) that ensures production of $H_2O_2$, which then will initiate the oxidation of for instance $SCN^-$ or $I^-$ into antimicrobial reagents ($SCNO^-$ or $I_2$) by a peroxidase (usually lactoperoxidase). This enzymatic complex is known in nature from e.g. milk and saliva.

It is being utilised commercially as antimicrobial system in oral care products (mouth rinse, dentifrice, chewing gum) where it also can be combined with an amyloglucosidase to produce the glucose. These systems are also known in cosmetic products for preservation.

Antimicrobial systems comprising the combination of an oxidase and a peroxidase are know in the cleaning of contact lenses.

Other applications of oxidoreductases are the application of oxidases, peroxidases and laccases in oxidative hair dyeing.

Further, free radicals formed on the surface of the skin (and hair) known to be associated with the ageing process of the skin (spoilage of the hair).

The free radicals activate chain reactions that lead to

destruction of fatty membranes, collagen, and cells.
The application of free radical scavengers such as Superoxide dismutase into cosmetics is well-known(R.L. Goldemberg, DCI, Nov. 93, 48-52).

Protein disulfide isomerase (PDI) is also an oxidoreductase. It can be utilised for waving of hair (reduction and reoxidation of disulfide bonds in hair) and repair of spoiled hair (where the damage is mainly reduction of existing disulfide bonds).

4) Glucanases/Carbohydrases.
Plaque formed on the surface of teeth are composed mainly of polysaccharides. They stick to the surface of the teeth and the microorganisms. The polysaccharides are mainly α-1,6 bound glucose (dextran) and α-1,3 bound glucose (mutan). The application of different types of glucanases such as mutanase and dextranase helps hydrolysing the sticky matrix of plaque, making it easier to remove by mechanical action.

Also other kinds of biofilm for instance the biofilm formed in lens cases can be removed by the action of glucanases.

5) Antimicrobial polypeptides.
Antimicrobial polypeptides have widespread applications such as preservation of cosmetic products, anti-acne products, deodorants and shampoos.

Food and Feed

The polypeptides with reduced allergenicity according to the invention may further advantageously be used in food- and feedstuff. Specifically relevant polypeptides are enzymes selected from the group of proteases, β-glucanases, amylases, pectinases, α-galactosidases, phytases, xylanases and lipases.

Use of Zipper domains

Finally the invention relates to the use of Zipper domains for reducing allergenicity of polypeptides and may be any molecules capable of self-oligomerizing microbially expressed

polyp ptides.

Examples of a number of Zipper domains have already been described previously.

In an embodiment the Zipper domain is a Leucine Zipper.

The Leucine Zipper may be any known Leucine Zipper which, grafted to a polypeptide, is capable of self-oligomerizing as a result of association of parallel α-helical coils of two or more Leucine Zippers.

In a specific embodiment the Leucine Zipper is the yeast transcriptional factor GCN4 or modifications thereof.

If it is desired to obtain a hetero-dimeric molecule the Zipper domain may advantageously be a Fos Leucine Zipper and a Jun Leucine Zipper.

In another embodiment of the invention the Zipper domain is a four helical bundle or a modification thereof.

The Zipper domains may advantageously be used for reducing allergenicity of polypeptides in detergents, household articles, agrochemicals, personal care products, cosmetics, toiletry, pharmaceuticals, composition used for treating textiles, food and feed etc.

Specificially the polypeptides comprising at least one Zipper domain may advantageously be used in compositions and/or in context with *e.g.* the industrial applications previously described.

The present invention is further illustrated in the following exampl s which should not, in any manner, be considered to limit the scope of the present invention.

**METHODS AND MATERIALS**

Host cells:

*Escherichia coli* JM105 (Yanisch-Perron et al. Gene, 33, p. 103-119, 1985)

*Epicurian coli*® XL1-Blue Cells (Stratagene Cloning Systems, Ca., USA)

*Escherichia coli* MC1061 (Casadaban, M.J. et al., J. Mol.Biol. 138, p. 179-207, 1980).

Vector:

The pFab3 expression vector is the ancestor of pFab4 (Ørum, H. et al., Nucleic Acids Research, 21, p. 4491-4498, 1993). The vector contains a pelB signal sequence (Lei et al., J. of Bacteriol, vol. 169, p. 4379-4383, 1987) which is under control of the inducible lacZ promoter. A SfiI site in the pelB signal makes it possible to clone the desired sequence, so that the geneproduct will be expressed In Frame with the signal sequence.

Parts of the pFab3 vector was not relevant for this study wherefor it was removed by SfiI and XmaI digestion. These sites were then used as sites for introducing the PCR fragment as described below. In contrast to the referred pFab4, pFab3 contains 131 bp region between the start codons of the lacZ and the pelB signal.

Primers:

A-termamyl (SEQ ID NO 9):

5'-CA GTC ACA GAT CCT CGC GAA TTG GCC CAG CCG GCC ATG GCC GCA AAT CTT AAT GGG ACG CTG ATG-3'

B-termamyl (SEQ ID NO 10):

5'- CAT TCG CGA GGA CCC GGG CGG GGT GGA CGG TTT CGG TCT TTG AAC ATA AAT TGA AAC CGA CCC-3'

The underlined nucleotides correspond to the termamyl sequence.

Primer A-termamyl also includes the SfiI restriction site and the last two codons of the pelB signal. Primer B-termamyl includes a linker sequenc (a short hinge domain of IgG3)(Plückthun, A. et al., Biochemistry, 31, p. 1579-1584, 1992) and a XmaI cloning site.

Plasmids:
pDN1528 (PCT/DK94/00370)

Signal sequence:
pelB (Lei et al., J. of Bacteriol, vol. 169, p. 4379-4383, 1987)

Linker sequence:
Part of $IgG_3$ hinge (Plückthun, A. et al., Biochemistry, 31, p. 1579-1584, 1992)

**Materials:**
fmol™ DNA-sequencing system (Cat.:#Q4100, Promega Corporation, WI, USA).
α-amylase EPS assay (Cat.:#1442295, Boehringer Mannheim GmbH, Mannheim, Germany)

Enzymes:
Termamyl® (available from Novo Nordisk A/S)
SfiI (Cat.:#R6391, Promega Corporation, WI, USA)
XmaI (Cat.:#R6491, Promega Corporation, WI, USA)
SacI (Cat.:#R6061, Promega Corporation, WI, USA)
T4-DNA ligase (Cat.:#M1801, Promega Corporation, WI, USA)
AmpliTaq® DNA Polymerase (PartNo.:N801-0060, Perkin Elmer, Roche Molecular Systems, New Jersey, USA.)

Solutions:
PCR reaction buffer: dNTP(0.25mM of each), $MgCl_2$ 2.5mM and 1x PCR reaction buff r-II (PartNo.:N808-0009, Perkin Elm r, Roche Molecular Systems, New Jersey, USA.)
T4-DNA ligase buffer (Cat.:#M1801, Promega Corporation, WI, USA)

SOC medium (Sambrook, J. et al., 1989, Molecular Cloning. A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory, New York, USA )

2xTY medium (Ausubel, F.M. et al. (Editors), 1994

LB-agar Current Protocols in Molecular Biology, John

LB-medium Wiley & Sons, Inc. and Greene Publishing Associates, Inc., New York, USA)

PEG-8000 (Cat.:#P2139, Sigma Chemical Company, MO, USA).

PBS tween 20 Ausubel, F.M. et al. (Editors), 1994

Alkaline phosphatase Buffer (pH=9.0)

| | |
|---|---|
| NaCl | 5.844 g |
| $MgCl_2,6H_2O$ | 1.02 g |
| Diethanol amine | 10.51 g |

The pH is adjusted to 9.0 with HCl, and Milli-Q water is applied to 1 litre.

Stop-solution

| | |
|---|---|
| EDTA, disodium | 74.44 g |
| $K_2HPO_4$ | 174.2 g |
| $NAH_3$ | 0.2 g |

The pH is adjusted to 10 with about 22.5 g KOH in Milli-Q water to 1 litre.

Equipment:

Bio-Rad *E. coli* pulser(#165-2103,Bio-Rad Laboratories, Ca., USA)

Horizon 11.14 Agarose Gel apparatus. (#580-1068IL, Life Technologies, Inc.,MD, USA).

Applied Biosystems 394 DNA/RNA synthesizer (Applied Biosystems, CA, USA).

Thermocycler Varius V 45(Hans Landgraf, GmbH, Langenhagen, Germany.)

Mini-PROTEAN II Electrophoresis Cell (#165-2940,Bio-Rad Laboratories, Ca., USA)

The Semi Dry Electroblotter (JKA-Biotech, Denmark)

HiTrap™ chelating column (Code no. 17-0409-01, Pharmacia LKB, Biotechnology AB, Uppsala, Sweden)

ELISA reader: Ceres 900 HDi

**Methods:**

All general techniques are performed according to Sambrook, J. et al., Molecular Cloning. A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory, New York, USA, 1989, and/or according to Ausubel, F.M. et al. (Editors), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. and Greene Publishing Associates, Inc.,New York, USA, 1994)

Geneclean II procedure (BIO 101, Inc., CA, USA).

PCR amplification of the gene encoding Termamyl®.

The PCR reaction is performed in 50 µl volume PCR reaction-buffer, 1 µM of each primer A- and B-termamyl and 10 ng of the DNA template.

Reaction mixtures are overlaid with mineral oil and kept at 94°C for 5 minutes. Then 0.5 µl of AmpliTaq® (5 U/µl) is added. The mixtures are kept at annealing temperature 70°C for 5 minutes and at elongation temperature 72°C for 2 minutes.

After this initial incubation the mixtures cycle 30 times (94°C 1 minute, 70°C 1 minute, 72°C 1 minute) followed by incubation at 72°C for 10 minutes using a PCR Thermocycler Varius V 45.

Preparation of a sequence encoding Leucine Zipper from GCN4 and linker

The four oligonucleotides are synthesized on Applied Biosystems 394 DNA/RNA synthesizer according to protocols of the supplier. After synthesis the oligonucleotides are purified using denaturing polyacrylamide gel electrophoresis according to (Ausubel, F.M. et al., supra, 1994). 20 pmol of each oligonucleotide are mixed in a total volume of 40 µl of 100 mM NaCl, annealed by incubation at 95°C for 5 minutes and cooled slowly to 16°C over a period of 3 hours, this annealing mixture is used in ligation. The four oligonucleotides are as follows:

Antisense Zip Cys (1) (XmaI-SacI) (SEQ ID NO 3)

5′-CA GCC CCC ACA GCC CCC ACG TTC ACC AAC AAG CTT TTT CAG ACG AGC AAC TTC GTT TTC CAG GTG GTA G-3′

Antisense Zip (2). (XmaI-SacI) (SEQ ID NO 4)

5′-P-TT TTT GGA CAG CAG TTC TTC AAC TTT GTC TTC CAG CTG TTT CAT TCG CGA GGA C-3′

Sense Zip (1). (XmaI-SacI) (SEQ ID NO 5)

5′-CCG GGT CCT CGC GAA TGA AAC AGC TGG AAG ACA AAG TTG AAG AAC TGC TGT CCA AAA ACT ACC ACC-3′

Sense Zip Cys (2). (XmaI-SacI) (SEQ ID NO 6)

5′-P-TG GAA AAC GAA GTT GCT CGT CTG AAA AAG CTT GTT GGT GAA CGT GGG GGC TGT GGG GGC TGA GCT-3′

P indicates oligonucleotides with a phosphoryl group at the 5′-ends.

Transformation of XL-1 blue *E. coli*

The transformation is carried out by electroporation. For this purpose *Epicurian coli*® XL1-Blue Electroporation competent Cells are used, 3 µl of ligated DNA is used per 80 µl of cells. The electroporation is performed using a Bio-Rad *E. coli* pulser set at 25 µF, 2.5 kV and 200 Ohms.

Transformation of *E.coli* JM105

Transformation of *E. coli* JM105 is done by adding 3 µl of the ligation mixture per 100 µl of heat-shock competent cells. The preparation and transformation of the cells are made essentially as described in (Sambrook et al. 1989, supra.)

Expression and isolation of periplasmic polypeptides.

The expression of Termamyl -dimer in *E. coli* JM105 is done as follows. An overnight culture of JM105 harbouring the pAZ-1 plasmid in 2XTY medium with 100 µg/ml Ampicillin and 1% D(+)Glucose is prepared by transferring a single colony to the

media and incubating this at 37°C for 16 hours with vigorously shaking. 100 µl of this is used as starter culture of 100 ml 2XTY medium with 100 µg/ml Ampicillin and 0.1% D(+)-Glucose, which in a 1 L shake flask is incubated at 37°C with vigorously shaking. When OD550=1.0 is reached, the temperature is adjusted to 30°C and the expression is induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of within 1-5 mM. The induction is carried out for 5 hours, then the cells are pelleted by gentle centrifugation and given an osmotic shock for releasing the polypeptides present in the periplasmic space of the cells. This can be performed according to the procedure of Neu, H.C. and Heppel, L.A., J. Biol. Chem. 240, p. 3685-3692, 1965.

Characterization of expressed Termamyl®-Zipper dimer

The periplasmic fraction of induced and non-induced cells harbouring the pAZ-1 plasmid are analyzed on an SDS-page gel 4-20% Acrylamide (Laemmeli, Nature, 227, p. 680, 1970) using the Mini-Protean II (Bio-Rad Laboratories, Richmond, Ca, USA). Samples are run with or without reducing agent dithiothreitol. Dithiothreitol (DTT) also termed Clelands reagent is a reducing agent capable of quantitatively reducing disulfide bonds (Cleland, W.W., Biochemistry, 3, p. 480, 1964). One gel is stained with Coomassie Brilliant Blue G250 according to (Neuhoff, et al., Electrophoresis, 9, p. 255-262, 1988) and another gel is used for blotting the polypeptides onto a PVDF membrane Imobillin-P®(Cat.:#IPVH 20200, Millipore Corporation, MA, USA) using The Semidry Electroblotter. The membranes are probed using anti-Termamyl antibodies raised in rabbits against the Termamyl® as the primary antibody (as described in Ausubel, F.M. et al. chapter 11 section 12 and 13, supra, 1994) and anti-rabbit IgG horseradish peroxidase conjugated (Cat.:L42007, medac, GmbH, Hamburg, Germany) as secondary antibody. The polypeptides are detected with ECL™ Western blotting detection reagent (Cat.:#RPN2106, Amersham Int., Buckinghamshire, England) and recording the light emission on ordinary X-ray film.

Purification of Termamyl Zipper dimer

The expressed Termamyl Zipper dimer was purified from the fermentation broth. This was done using the attached poly-His tail as the affinity tag. More specifically the purification was performed using a 5 ml HiTrap™ chelating column according to the recommodations of the supplier. Further details concerning the IMAC (Immobbilized Metal Affinity Chromatography) procedure is describe in Yip et al., (1994), Molecular Biotechnology, vol. 1, p. 151-164; Fatiadi et al., (1987), CRC Critical Rev. Anal. Chem. 18, p. 1-44.

Protein determination

After purification and dialysis of the Termamyl® Zipper dimer sample, spectral measurements were performed. The optical density of the sample at 280 nm were used to calculate the protein concentration of the sample. For this purpose the Lambert Beer law was used together with the calculated protein extinction coefficient of the Termamyl® Zipper dimer using the principals for this as described by Gill et al, Analytical Biochemistry, 182, p. 319-326, 1989.

ELISA procedure for determination of $IgG_1$ positive guinea pigs

ELISA microtiter plates are coated with rabbit anti-Termamyl® AAN 4080 K 452-453 1:4000 in carbonate buffer and incubated over night at 4°C. The next day the plates is blocked with 2% BSA for 1 hour and washes 3 times with PBS tween 20. Termamyl® PPX 3328 1 µg enzyme protein/ml is applied to the plates, incubated for 1 hour and then washed 3 times with PBS tween 20.

All guinea pig samples are applied to the ELISA plates with 25 µl sera and 25 µl PBS buffer, incubated for 3 hours and washed 3 times with PBS tween 20.

Then goat anti-guinea pig $IgG_1$ (diluted to 1:4000 in PBS buffer) is applied to the plates, incubated for 1 hour and wash d 3 times with PBS tween 20. Alkaline phosphatase rabbit anti-goat is applied (diluted to 1:8000) and incubated for 1 hour, washed 2 times in PBS tween 20 and 1 time with diethanol amine buffer.

The alkaline phophatase is developed using p-nitrophenyl phosphate for 30 minut s at 37°C and stopp d with calcium/sodium buffer comprising EDTA (pH=10) and read at OD 405/650 using a ELISA reader.

6 double blinds are included on all ELISA plates.

Positive and negative sera values are calculated as the avarage blind values added 2 times the standard deviation. This gives an accuracy of 95%.

The test is described more thoroughly in ED-9515452 available on request from Novo Nordisk A/S.

**EXAMPLES**

**EXAMPLE 1**

PCR amplification and cloning of the gene encoding Termamyl®.
The primers A-termamyl and B-termamyl were designed and synthesized on Applied Biosystems 394 DNA/RNA synthesizer.

The Termamyl® encoding gene was PCR amplified using the plasmid pDN1528 containing the gene encoding Termamyl® as a template.

The PCR product, a 1.5 kb fragment, was purified by preparative agarose electrophoresis followed by the Geneclean-II procedure (BIO 101, Inc., Ca., USA).

**EXAMPLE 2**

Cloning of PCR product

The purified 1.5 kb DNA fragment containing the sequence encoding Termamyl was digested with 10 U of SfiI per μg DNA at 50°C for 2 hours with the reaction mixture overlaid by mineral oil.

Following the Geneclean-II procedure the DNA fragment was further digested for 2 hours at 37°C with 10 U XmaI per µg DNA. The digested DNA was again purified using Geneclean-II procedures and finally ligated to the prepared SfiI and XmaI digested pFab3. The 10 µl ligation mixture included 0.2 µg of insert DNA and 0.2 µg of digested vector pFab3. The ligation was performed for 2 hours at 16°C and 14 hours at 4°C with 1 U of T4-DNA ligase.

The ligated material was used to transform *Epicurian coli*® XL1-Blue Electroporation competent Cells as described above.

EXAMPLE 3

Identification of correct clones

Immediately after the electroporation 1 ml of freshly made SOC medium was added and the transformed cells were vigorously shaken for 1 hour at 37°C, plated onto LB-agar plates containing 100 µg/ml Ampicillin and 12.5 µg/ml Tetracyclin and incubated overnight at 37°C. The next day clones were picked randomly, transferred to 14 ml polypropylene tubes containing 2 ml of LB medium containing 100 µg/ml Ampicillin and 12.5 µg/ml Tetracyclin. After overnight incubation at 37°C and shaking at 250 rpm, plasmid DNA minipreps were prepared according to Sambrook et al., supra, 1989. The isolated plasmid DNA was analyzed by digestion with SfiI and XmaI and the digested plasmid DNA was analyzed on 1% agarose, 1xTBE gel.

The appearance of a DNA fragment of 1.5 kb indicated the existence of clones containing the correct fragment size. Further verification of the cloned gene was done as DNA sequencing using the fmol™ DNA-sequencing system from Promega Corporation. A construct with the correct DNA sequence of Termamyl was named pAZ-½.

EXAMPLE 4

Leucine Zipper

Introduction of a sequence encoding the Leucine Zipper of GCN4 a Yeast transcriptional activator (O'Shea et al. Science, 243, p. 538-542, 1989) and also encoding a flexible C-terminal extension peptide containing a Cysteine amino acid residue was carried out using four overlapping oligonucleotides (see methods section).

For ligation 5 µl of the annealing mixture was added to the T4-DNA ligase buffer to which was added 5% PEG-8000, 1 Unit of T4-DNA ligase and 0.1 pmol of XmaI and SacI digested pAZ-½.

The ligated material was used to transform *E. coli* JM105 as described above.

EXAMPLE 5

Identification of clones containing the Termamyl®-Leucine Zipper construct

Immediately after the transformation 1 ml of freshly made SOC medium was added and the cells were vigorously shaken for 1 hour at 37°C, plated onto selective plates and incubated overnight at 37°C.

The next day clones were picked randomly, transferred to 14 ml polypropylene tubes containing 2 ml of LB medium containing 100 µg/ml Ampicillin. After overnight incubation at 37°C and shaking at 250 rpm, plasmid DNA minipreps were prepared according to (Sambrook et al., supra, 1989). The isolated plasmid DNA was analyzed by digestion with NruI (introduced by the Leucine Zipper DNA fragment), the digested and non-digested plasmid DNA were analyz d on 1% agarose, 1xTBE gel. The appearance of linearized plasmid of 4606 bp in NruI dig sted sampl s indicated th existence of clon s comprising the Leucine Zipper fragment. Further v rification of the cloned fragment was done by sequ-

encing using the fmol™ DNA-sequencing system from Promega Corporation.

EXAMPLE 6

Expression of dimerized Termamyl®

JM105 cells harbouring the plasmid pAZ-1 were induced to express the fusion polypeptide *pelB* signal-Termamyl®-Linker-Leucine Zipper as described above. Each fusion polypeptide dimerize with other identical fusion polypeptides during expression. Cells were given osmotic shock in order to release proteins present in the periplasma. Aliquots of the isolate from both induced and non-induced cells were analyzed on SDS-PAGE. Samples were analyzed under reducing (sample with DTT) and non-reducing (sample without DTT) conditions. Polypeptide bands were visualised by staining with Coomassie Blue dye. Non-reduced samples of induced cells showed a distinct band at approximately 120 kDa (see figure 3) a band not present in samples of non-induced cells. Reduced samples from induced cells showed a distinct band at approximately 60 kDa, while at the same time no band was seen at 120 kDa (see figure 4). In samples of non-induced cells no 60 kDa band were seen. A gel similar to the above were used to transfer the proteins to a PVDF membrane via a Western blot procedure, as described above. The 120 kDa band of non-reduced samples was specifically recognized as Termamyl® confirming the expression of Termamyl as a dimer (see figure 5).

EXAMPLE 7

α-amylase activity of unpurified Termamyl-Dimer.

As a test for α-amylase activity of the Termamyl -dimer, a sample of the periplasmic isolate was analyzed. It was estimated by electrophoretic analysis that this sample contained about 0.5 mg/ml of Termamyl -dimer. Dilutions of the sample were tested in an α-amylase assay (see the Materials and

Methods section) and compar d to dilutions of Termamyl of known activity. This assay revealed that the dimer retained more than 50% of the wild-type activity.

EXAMPLE 8

Introduction of a purification tag

To obtain a more pure expression product a purification tag was introduced at the C-terminal part of the Termamyl®-Zipper protein as an in-frame insertion between the Xma I and SacI sites of pAZ-½. This resulted in a nucleotide sequence encoding the Termamyl®-Zipper protein with a C-terminal tail consisiting of a Factor Xa site and the amino acid sequence His-His-His (see sequence data). Four oligonucleotides were used for this purpose (See below).

The four oligonucleotides were synthesized on Applied Biosystems 394 DNA/RNA synthesizer according to protocols of the supplier. After synthesis, the oligonucleotides were purified using denaturing polyacrylamide gel electrophoresis according to (Ausubel, F.M. et al., supra, 1994).

20 pmol of each oligonucleotide were mixed with a total volume of 40 µl of 100 mM NaCl, annealed by incubation at 95°C for 5 minutes and cooled slowly to 16°C over a period of 3 hours. This annealing mixture was used for ligation. The four oligonucleotides were as follows:

Antisense Zip-Xa-His (XmaI-SacI) (SEQ ID NO 7)

5′-CA ATG GTG ATG ACG ACC TTC GAT GCC CCC ACA GCC CCC ACG TTC ACC AAC AAG CTT TTT CAG ACG AGC AAC TTC GTT TTC CAG GTG GTA G-3′

Antisense Zip, (XmaI-SacI) (SEQ ID NO 4)

5′-P-TT TTT GGA CAG CAG TTC TTC AAC TTT GTC TTC CAG CTG TTT CAT TCG CGA GGA C-3′

Sense Zip, (XmaI-SacI) (SEQ ID NO 5)

5'-CCG GGT CCT CGC GAA TGA AAC AGC TGG AAG ACA AAG TTG AAG AAC TGC TGT CCA AAA ACT ACC ACC-3'

Sense Zip-Xa-His, (XmaI-SacI) (SEQ ID NO 8)

5'-P-TG GAA AAC GAA GTT GCT CGT CTG AAA AAG CTT GTT GGT GAA CGT GGG GGC TGT GGG GGC ATC GAA GGT CGT CAT CAC CAT TGA GCT-3'

P indicates oligonucleotides with a phosphoryl group at the 5'-ends.

The oligonucleotides were designed to have extruding overhangs when hybridized: one matching the XmaI site and one matching the SacI site of pAZ-½. The preperation of the pAZ-½ for ligation with the hybridized oligonucleotides, the actual ligation and transformation of competent *E. coli* JM105, were performed essentially as described in example 4 and in the Methods and Materials-section.

From this transformation positive clones were identified as described in Example 5 and again the DNA sequence was verified by DNA sequencing. Further, dimer Termamyl® was expressed as described in Example 6.

EXAMPLE 9

Allergenicity trails of dimer Termamyl®

20 Dunkin Hartley guinea pigs were exposed to 1.0 µg monomer Termamyl® and 1.0 µg dimer Termamyl® by intratracheal dosage as described ED-9513462 available on request from Novo Nordisk A/S.

All guinea pigs were tested for the production of $IgG_1$ (indicating an allergic response) during 8 days using the ELISA procedur described above.

Figure 6 shows the number of Dunkin Hartley guinea pigs found $IgG_1$ positive during the trail period.

It can be seen from figure 6 the number of guinea pigs being $IgG_1$ positive at any time during the trial period is reduced for the dimer Termamyl® in comparison to the monomer Termamyl®. This proves that the allergenicity of Termamyl® can be reduced by coupling Termamyl® to a Zipper domain.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: A process for the production of polypeptides

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1593 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1593

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC      48
Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
 1               5                  10                  15

AAT GAC GGC CAA CAT TGG AGG CGT TTG CAA AAC GAC TCG GCA TAT TTG      96
Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu
            20                  25                  30

GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA     144
Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45
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ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA      192
Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
     50                  55                  60

GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA      240
Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
 65                  70                  75                  80

GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATC AAC      288
Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
                 85                  90                  95

GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC      336
Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
            100                 105                 110

GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA      384
Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
        115                 120                 125

ATT TCA GGA GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG      432
Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
    130                 135                 140

GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT      480
Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145                 150                 155                 160

GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG      528
Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
                165                 170                 175

TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC      576
Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
            180                 185                 190

TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC      624
Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
        195                 200                 205

GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA      672
Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
    210                 215                 220

TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT      720
Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225                 230                 235                 240

TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG      768
Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
                245                 250                 255

TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC      816
Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
            260                 265                 270
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TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT          864
Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
        275                 280                 285

CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG          912
His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
    290                 295                 300

AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG          960
Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305                 310                 315                 320

GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG         1008
Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335

TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC         1056
Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350

ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG         1104
Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365

ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT         1152
Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
    370                 375                 380

GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT         1200
Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385                 390                 395                 400

GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC         1248
Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415

AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC         1296
Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA         1344
Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
        435                 440                 445

TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG         1392
Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
    450                 455                 460

GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA ATT TAT         1440
Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465                 470                 475                 480

GTT CAA AGA CCG AAA CCG TCC ACC CCG CCC GGG TCC TCG CGA ATG AAA         1488
Val Gln Arg Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Met Lys
                485                 490                 495
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CAG CTG GAA GAC AAA GTT GAA GAA CTG CTG TCC AAA AAC TAC CAC CTG      1536
Gln Leu Glu Asp Lys Val Glu Glu Leu Ser Lys Asn Tyr His Leu
            500                 505                 510

GAA AAC GAA GTT GCT CGT CTG AAA AAG CTT GTT GGT GAA CGT GGG GGC      1584
Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg Gly Gly
        515                 520                 525

TGT GGG GGC                                                         1593
Cys Gly Gly
    530
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 531 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
 1               5                  10                  15

Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu
            20                  25                  30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
    50                  55                  60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65                  70                  75                  80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
                85                  90                  95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
            100                 105                 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
        115                 120                 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
    130                 135                 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145                 150                 155                 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
                165                 170                 175
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Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
180 185 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
195 200 205

Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
210 215 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225 230 235 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
245 250 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
260 265 270

Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
275 280 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
290 295 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305 310 315 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
325 330 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
340 345 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
355 360 365

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
370 375 380

Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385 390 395 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
405 410 415

Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
420 425 430

Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
435 440 445

Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
450 455 460

Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465 470 475 480

Val Gln Arg Pro Lys Pro Ser Thr Pro Pro Gly Ser Ser Arg Met Lys
485 490 495

Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr His Leu
500 505 510

Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg Gly Gly
515 520 525

Cys Gly Gly
530

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CAGCCCCCAC AGCCCCCACG TTCACCAACA AGCTTTTTCA GACGAGCAAC TTCGTTTTCC 60

AGGTGGTAG 69

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTTTTGGACA GCAGTTCTTC AACTTTGTCT TCCAGCTGTT TCATTCGCGA GGAC 54

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCGGGTCCTC GCGAATGAAA CAGCTGGAAG ACAAAGTTGA AGAACTGCTG TCCAAAAACT 60

ACCACC 66

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGGAAAACGA AGTTGCTCGT CTGAAAAAGC TTGTTGGTGA ACGTGGGGGC TGTGGGGGCT 60

GAGCT 65

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CAATGGTGAT GACGACCTTC GATGCCCCCA CAGCCCCCAC GTTCACCAAC AAGCTTTTTC 60

AGACGAGCAA CTTCGTTTTC CAGGTGGTAG 90

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 86 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TGGAAAACGA AGTTGCTCGT CTGAAAAAGC TTGTTGGTGA ACGTGGGGGC TGTGGGGGCA 60

TCGAAGGTCG TCATCACCAT TGAGCT 86

(2) INFORMATION FOR SEQ ID NO: 9

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 65 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CAGTCACAGA TCCTCGCGAA TTGGCCCAGC CGGCCATGGC CGCAAATCTT AATGGGACGC 60

TGATG 65

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 63 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CATTCGCGAG GACCCGGGCG GGGTGGACGG TTTCGGTCTT TGAACATAAA TTGAAACCGA 60

CCC 63

**PATENT CLAIMS**

1. A process for producing a polypeptide with reduced allergenicity, by
a) fermenting a microorganism capable of producing said polypeptide, and
b) recovering said polypeptide in substantially pure form, wherein said microorganism is modified in a manner whereby the expressed polypeptide molecules self-oligomerize.

2. The process according to claim 1, wherein said microorganism is modified by the introduction of one or more DNA constructs comprising at least one DNA sequence coding for at least one polypeptide and at least one Zipper domain operably linked to each other.

3. The process according to claim 2, wherein the Zipper domain is an α-helical bundle comprises from two, three, four, five, six or seven helices

4. The process according to claims 1 and 2, wherein the said Zipper domain is a Polar Zipper of poly(L-glutamine) repeats.

5. The process according to any of the claims 2 to 3, wherein the Zipper domain comprises an amphiphilic helical bundle.

6. The process according to any of claims 2, 3 and 5, wherein the Zipper domain is a Leucine Zipper or a modification thereof.

7. The process according to claim 6, wherein the Zipper domain results in the formation of a hetero-dimer.

8. The process according to claim 7, wherein one Leucine Zipper is a Fos Leucine Zipper and the other is a Jun Leucine Zipper.

9. The process according to any of the claims 6 to 8, wherein

a cysteine is includ d in the Leucine Zipper.

10. The process according to any of claims 2, 3 and 5, wher in the Zipper domain is an antiparallel four-helical bundle or a modification thereof.

11. The process according to any of claims 1 to 10, wherein said DNA construct comprises an operably inserted linker sequence between the DNA sequence coding for said polypeptide and the DNA sequence coding for said Zipper domain.

12. The process according to any of the claims 1 to 11, wherein the DNA sequence encodes an enzyme.

13. The process according to claim 12, wherein the DNA sequence encodes at least one enzyme selected from the group comprising proteases (metallo, acid, neutral or alkaline), lipases, cellulases, amylases, lyases, xylanases, pectinases, pullulanase, polygalacturonases, oxidases, laccases, oxidoreductases, transglutaminases, α-galactosidases, phytases and peroxidases

14. The process according to any of claims 1 to 13, wherein the DNA sequence encodes polypeptides having a molecular weight of about 5 kDa to 150 kDa, preferably from 20 kDa to 100 kDa, especially from 20 kDa to 80 kDa.

15. The process according to any of claims 1 to 14, wherein the enzyme is Termamyl®.

16. The process according to any of claims 1 to 15, wherein the oligomerization is a dimerization.

17. The process according to any of claims 1 to 15, wherein the oligomerization is a trimerization.

18. The process according to any of claims 1 to 15, wherein the oligomerization is an tetramerization.

19. Th process according to claim 1 to 18, wherein the microorganism is a bacterium, a yeast or a filamentous fungus.

20. The process according to claim 19, wherein said bacterium is selected from the group comprising grampositive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans*, *S. murinus* or *S. griseus*, or gramnegative bacteria such as *Escherichia coli*.

21. The process according to claim 20, wherein the host cell is *B. licheniformis* or *E. coli*.

22. The process according to claim 19, wherein said yeast is selected from the group comprising *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*, or cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, such as *H. polymorpha*, or *Pichia*, in particular *P. pastoris*.

23. The process according to claim 19, wherein said filamentous fungus is selected from the group comprising *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger* or *F. oxysporum*.

24. A DNA construct for producing polypeptides with reduced allergenicity comprising a DNA sequence encoding at least one polypeptide and at least one Zipper domain operably linked to each other.

25. The DNA construct according to claim 24, comprising a linker sequence inserted operably between DNA coding for the par nt polypeptide and DNA coding for said Zipper domain.

26. The DNA construct according to claims 24 and 25, comprising

a DNA sequence which when expressed exhibits at least one enzymatic activity.

27. The DNA construct according to claim 26, capable of expressing an enzyme selected from the group comprising proteases (metallo, acid, neutral or alkaline), lipases, cellulases, amylases, lyases, xylanases, pectinases, polygalacturonases, oxidases, laccases, oxidoreductases, transglutaminases, α-galactosidases, phytases or peroxidases.

28. The DNA construct according to any of claims 24 to 27, wherein the DNA sequence encodes polypeptides with a molecular weight of about 5 kDa to 150 kDa, preferably from 20 kDa to 100 kDa, especially from 20 kDa to 80 kDa.

29. The DNA construct according to any of claims 24 to 28, wherein the enzyme is Termamyl®.

30. The DNA construct according to any of claim 24 to 29, comprising the DNA sequence shown in SEQ ID NO 1.

31. A recombinant vector or transformation vehicle, comprising a DNA construct according to any of claims 24 to 30.

32. The vector according to claim 31, wherein said DNA construct is operably linked to a secretion signal.

33. The vector according to claims 31 and 32, wherein said DNA construct comprise a sequence encoding an affinity tag.

34. The vector according to any of claims 31 to 33, wherein said vector is the pAZ-1 plasmid.

35. A cell comprising a DNA construct according to any of claims 24 to 30 or a recombinant vector or expression vector according to any of claims 31 to 34.

36. The cell according to claim 35, wherein the cell is a

bacterium, a yeast or a filamentous fungus.

37. The cell according to claim 36, wherein said bacterium is selected from the group comprising grampositive bacteria such as strains of *Bacillus*, such as strains of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megaterium* or *B. thuringiensis*, or strains of *Streptomyces*, such as *S. lividans*, *S. murinus* or *S. griseus*, or gramnegative bacteria such as *Escherichia coli*.

38. The cell according to claim 37, wherein the cell is *B. licheniformis* or *E. coli*.

39. The cell according to claim 36, wherein said yeast cell is selected from the group comprising *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or *Saccharomyces kluyveri*, or cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, such as *H. polymorpha*, or *Pichia*, in particular *P. pastoris*.

40. The cell according to claim 36, wherein the filamentous fungus is selected from the group comprising *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger* or *F. oxysporum*.

41. A microbially produced polypeptide with reduced allergenicity produced according to any of claims 1 to 23.

42. The polypeptide according to claim 41, comprising from 2 to 10 polypeptide molecules.

43. The polypeptide according to claim 42 is a dimer.

44. The polypeptide according to claim 42 is a trimer.

45. The polypeptide according to claim 42 is a tetramer.

46. Th polypeptide according to any of claims 41 to 45, exhibiting enzymatic activity.

47. The polypeptide according to claim 46, exhibiting at least one enzyme activity exhibited by enzymes selected from the group comprising proteases (metallo, acid, neutral or alkaline), lipases, cellulases, amylases, lyases, xylanases, pectinases, pullulanase, polygalacturonases, oxidases, laccases, oxidoreductases, transglutaminases, α-galactosidases, phytases and peroxidases

48. The polypeptide according to any of claims 41 to 47, wherein the monomeric polypeptide molecule has a molecular weight of about 5 kDa to 150 kDa, preferably from 20 kDa to 100 kDa, especially from 20 kDa to 80 kDa.

49. The polypeptide according to any of claims 41 to 48, wherein the enzyme exhibits α-amylase activity.

50. An oligomeric polypeptide with reduced allergenicity comprising at least one polypeptide bonded or linked to at least one Zipper domain which is coupled to at least one polypeptide bonded or linked to at least one Zipper domain.

51. The oligomeric polypeptide according to claim 50, wherein the Zipper domain comprises an α-helical bundle.

52. The oligomeric polypeptide according to claim 51, wherein the said α-helical bundle comprises two, three, four, five, six or seven helices.

53. The oligomeric polypeptide according to any of the claims 50 to 52, wherein the Zipper domain comprises an amphiphilic h lical bundle.

54. The oligomeric polypeptide according to claim 50, wherein the Zipper domain is a Polar Zipper of poly(L-glutamine) repeats or modifications thereof.

55. The oligomeric polypeptide according to any of claims 50 to 53, wherein the Zipper domain is a Leucine Zipper or a modification thereof.

56. The oligomeric polypeptide according to claims 54 and 55, wherein the Zipper domain oligomerization results in the formation of a hetero-dimer.

57. The oligomeric polypeptide according to claim 56, wherein one Leucine Zipper is a Fos Leucine Zipper and the other is a Jun Leucine Zipper.

58. The oligomeric polypeptide according to any of the claims 55 to 57, wherein a cysteine is included in the Leucine Zipper.

59. The oligomeric polypeptide according to any of claims 50 to 58, wherein the Zipper domain is an antiparallel four-helical bundle or a modification thereof.

60. The oligomeric polypeptide according to any of claims 50 to 59, wherein said DNA construct comprises an operably inserted linker sequence between the DNA sequence coding for said polypeptide and the DNA sequence coding for said Zipper domain.

61. The oligomeric polypeptide according to any of claims 50 to 60 wherein said polypeptide exhibits enzymatic activity.

62. The oligomeric polypeptide according to claims 61, wherein the polypeptide exhibits at least one enzyme activity exhibited by enzymes selected from the group comprising proteases (metallo, acidic, neutral or alkaline), lipases, cellulases, amylases, lyases, xylanases, pectinases, pullulanase, polygalacturonases, oxidas s, laccases, oxidoreductases, transglutaminases, α-galactosidases, phytases and peroxidases

63. The oligomeric polypeptide according to any of claims 61 to 62, wherein the monomeric polypeptide molecule has a molecular

weight of about 5 kDa to 150 kDa, preferably from 20 kDa to 100 kDa, especially from 20 kDa to 80 kDa.

64. The oligomeric polypeptide according to any of claims 61 to 63, wherein the enzyme exhibits α-amylase activity.

65. The oligomeric polypeptide according to any of claims 50 to 59, wherein said Zipper domain is linked to said polypeptide and the C-terminal of the polypeptide.

66. The oligomeric polypeptide according to any of claims 50 to 59, wherein said Zipper domain is linked to said polypeptide and the N-terminal of the polypeptide.

67. A composition comprising at least one polypeptide according to any of the claims 41 to 49 and/or at least one oligomeric polypeptides according to any of claims 50 to 66.

68. The composition according to claim 67, comprising ingredients normally used in detergents, household articles, agrochemicals, personal care products, cosmetics, toiletry, pharmaceuticals, composition use for treating textiles, food and/or feed.

69. Use of Zipper domains for reducing allergenicity of polypeptides.

70. The use according to claim 69, wherein said Zipper domains are used for oligomerizing polypeptide molecules.

71. The use according to claims 69 and 70, for a process according to any of claims 1 to 23 or in oligomeric polypeptides according to any of claims 50 to 66.

72. The use according to any of claims 69 to 71, wherein the Zipper domain comprises two, three, four, five, six or seven α-helical bundles.

73. The use according to any of claims 69 to 71, wherein the said Zipper domain is a Polar Zipper of poly(L-glutamin ) repeats or a modification thereof.

74. The use according to any of the claims 71 and 72, wherein said Zipper domain comprises an amphipathic helical bundle.

75. The use according to any of claims 71, 72 and 74, wherein said Zipper domain is a Leucine Zipper or a modification thereof.

76. The use according to claim 75, wherein the Leucine Zipper is a Fos-Jun Leucine Zipper.

77. The use according to any of the claims 75 and 76, wherein a cysteine is included in the Leucine Zipper.

78. The use according to any of claims 71, 72 and 74, wherein said Zipper domain is an antiparallel four-helical bundle or a modification thereof.

79. The use according to any of claims 69 to 78, in household articles.

80. The use according to any of claims 69 to 78, in detergents, including dishwashing detergents and soap bars.

81. The use according to any of claims 69 to 78, in personal care products.

82. The use according to claim 81, in oral care products including cleaning products for dentures and dentifrices.

83. The use according to claim 81, in skin care products including creams and lotions.

84. The use according to claim 81, in hair care or hair treatment products, including shampoos.

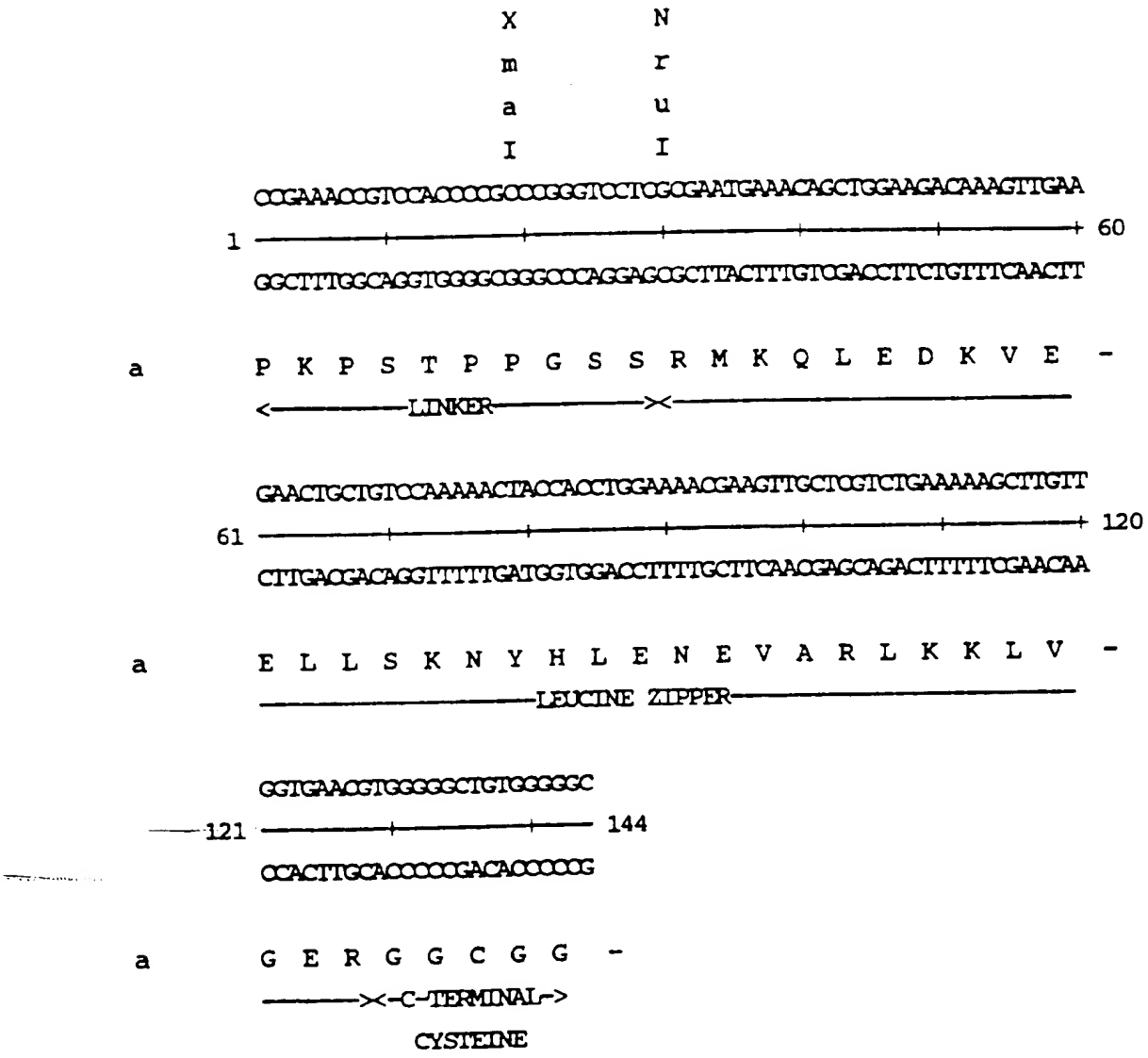
85. The use according to claim 81, in contact lens cleaning products.

86. The use according to any of claims 69 to 78, in cosmetics.

87. The use according to any of claims 69 to 78, in pharmaceuticals.

88. The use according to any of claims 69 to 78, in agrochemicals.

89. The use according to any of claims 69 to 78, in food and feed.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m r
a u
I I
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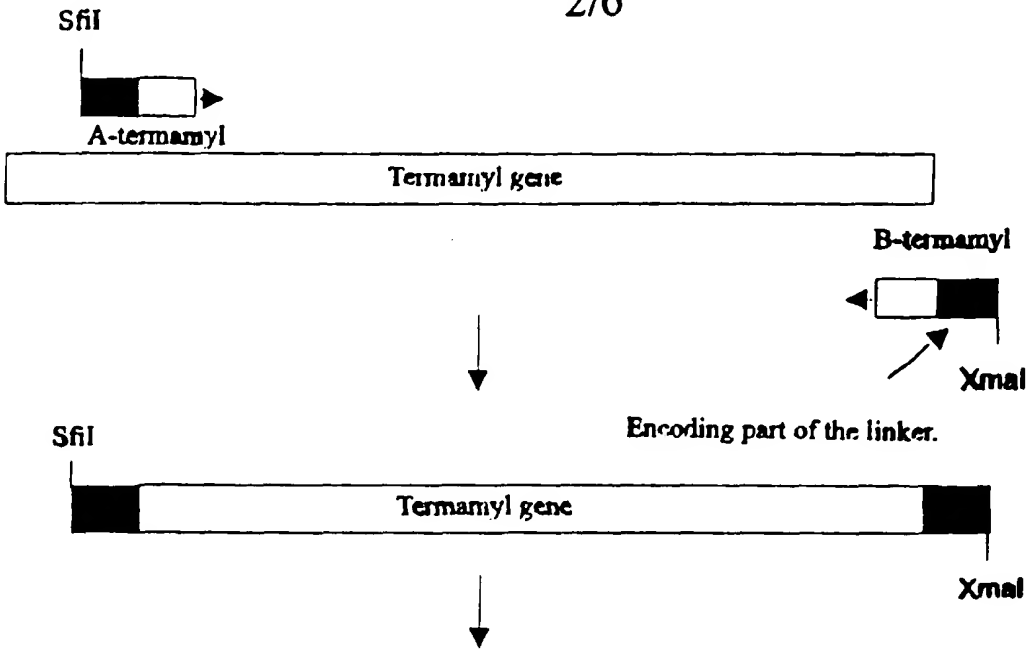
><-C-TERMINAL->
CYSTEINE

Figure 1




SfiI-XmaI digested fragment is inserted into SfiI-XmaI digested pFab3.

Linker encoding Leucine Zipper GCN4 is inserted through XmaI and SacI sites.

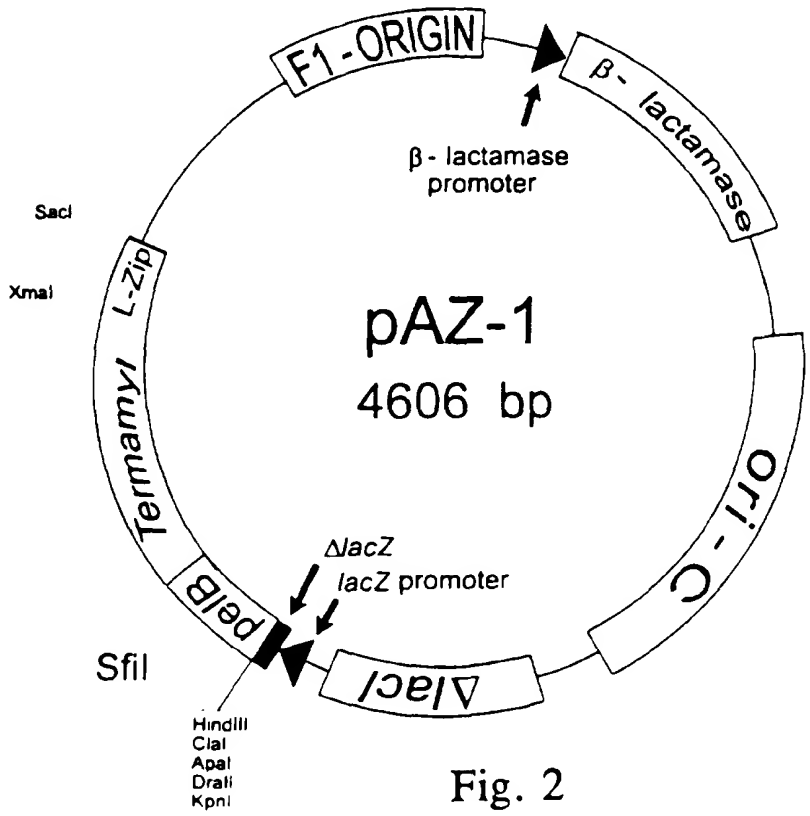



Fig. 2

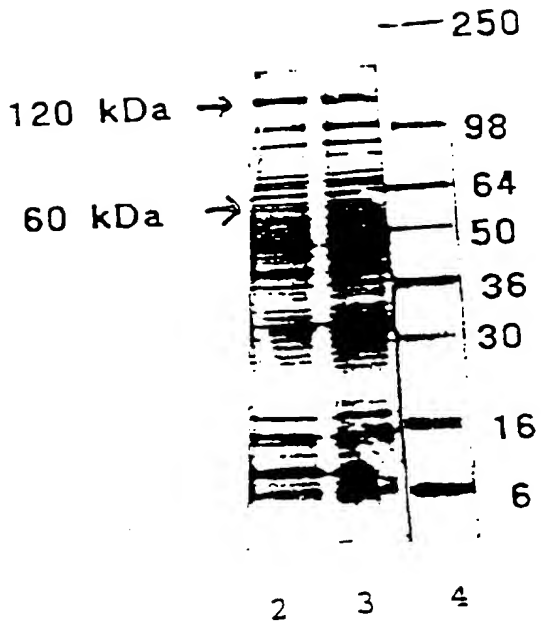



Figure 3

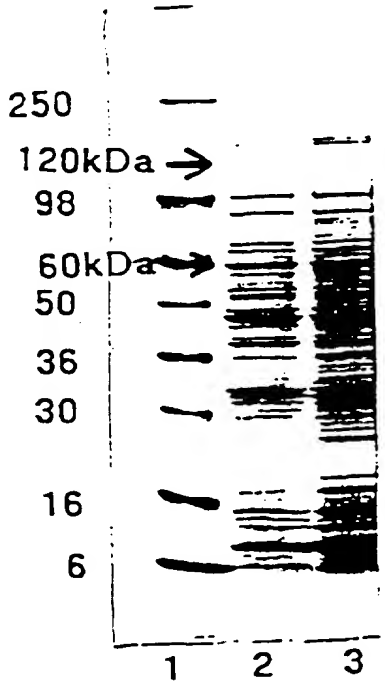



Figure 4

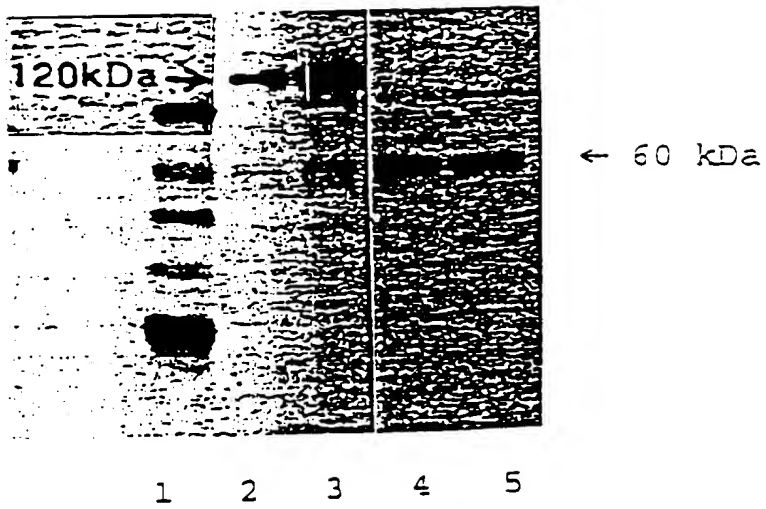



Figure 5

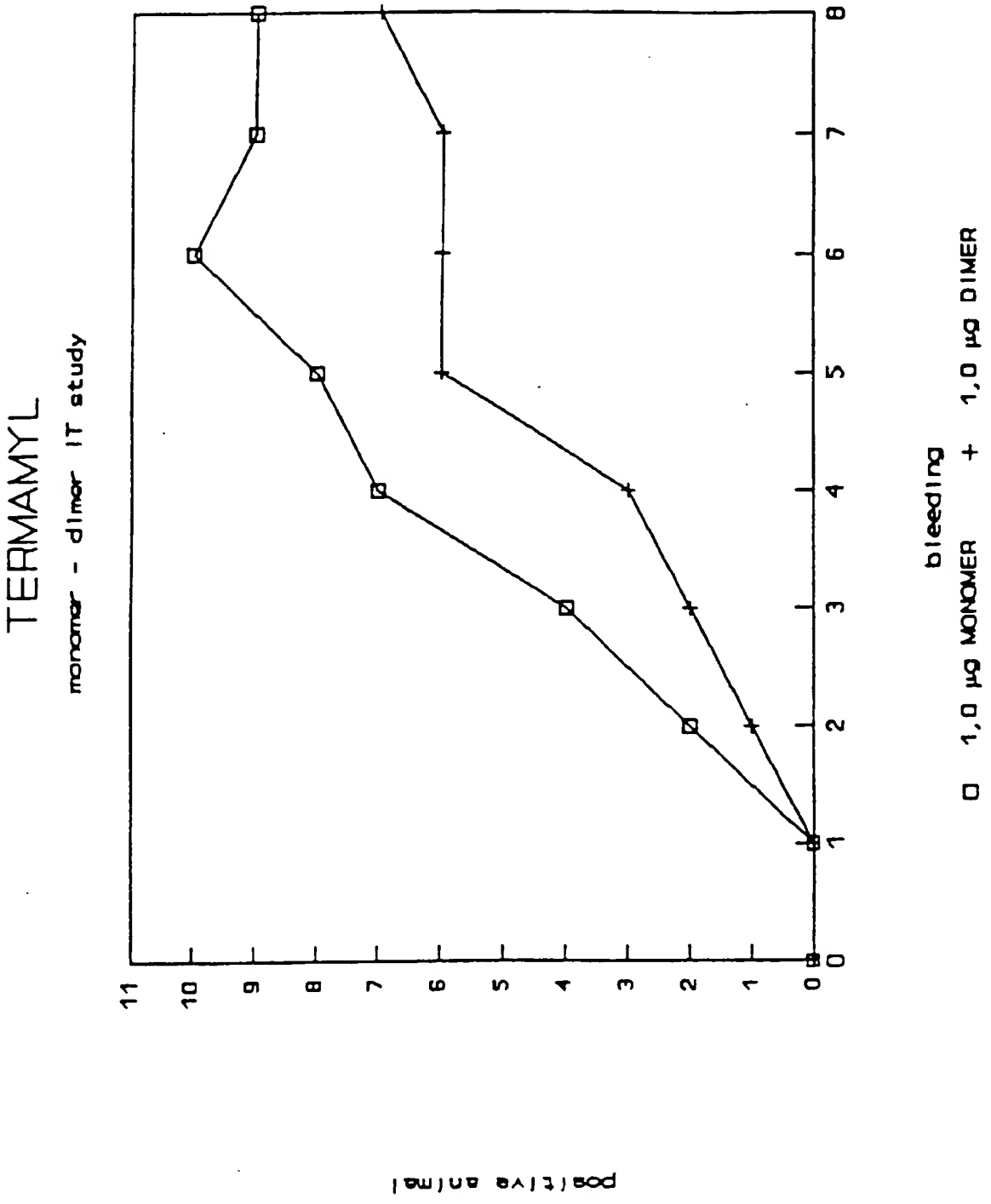

TERMAMYL
monomer - dimer IT study
bleeding
□ 1,0 µg MONOMER + 1,0 µg DIMER
positive animal
0 1 2 3 4 5 6 7 8
0 1 2 3 4 5 6 7 8 9 10 11

Fig. 6

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00463

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/62, C12N 9/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K, C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, US PATENTS FULLTEXT DATABASES, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9410308 A1 (IMMUNEX CORPORATION), 11 May 1994 (11.05.94), page 2, line 22 - line 24, the claims | 1-68 |
| Y | | 69-89 |
| | -- | |
| A | WO 9410191 A1 (NOVO NORDISK A/S), 11 May 1994 (11.05.94), page 3, line 33 - line 36; page 4, line 1 - line 8; page 4, line 26 - line 36, the claims and the abstract | 1-68 |
| Y | | 69-89 |
| | -- | |
| | -------- | |

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 March 1996 | 26 -03- 1996 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carolina Palmcrantz<br>Telephone No. +46 8 782 25 00 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

05/02/96

International application No.
PCT/DK 95/00463

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A1- 9410308 | 11/05/94 | NONE | |
| WO-A1- 9410191 | 11/05/94 | NONE | |